

Screening, Isolation and Production of Bioactive Compounds from Marine Actinomycetes

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ABSTRACT: Actinomycetes are a diverse group of Gram positive, free living, saprophytic, filamentous bacteria and are a major source for the production of antibiotics. The degradation and turnover of various materials are a continuous process mediated by the action of a variety of microorganisms. The Bay of Bengal an unexploited ecosystem that has as a source of novel and biologically potent marine derived compounds by several researchers. Around 23 000 bioactive secondary metabolites produced by microorganisms have been reported and over 10 000 of these compounds are produced by Actinomycetes, representing 45% of all bioactive microbial metabolites discovered.

KEYWORDS:- Actinomycetes, Gram positive, The degradation, secondary metabolites, bioactive compounds.

I. INTRODUCTION

Actinomycetes are a diverse group of Gram positive, free living, saprophytic, filamentous bacteria and are a major source for the production of antibiotics (Valli et al., 2012). They belong to the order Actinomycetales (Super kingdom: Bacteria, Phylum: Firmicutes, Class: Actinobacteria, Subclass: Actinobacteridae). They are found in soil, fresh water and marine water environments (Gebreselema et al., 2013). They have high G+C (>55%) content in their DNA. They are the best common source of antibiotics and provide approximately two-third of naturally occurring antibiotics, including many of medical importance (Okami & Hotta 1988). Actinobacteria were originally considered as an intermediate group between bacteria and fungi, but latter it has attained a distinct position (Pandey et al., 2004). Actinomycetes are the most economically and biotechnologically valuable prokaryotes and are responsible for the production of about half of the discovered secondary metabolites. In the recent times, they have been exploited successfully for their biologically potential secondary metabolites. They produce diverse group of antimicrobial metabolites notably glycopeptides, betalactams, aminoglycosides, polyenes, polyketides, macrolides, actinomycins and tetracyclins.

Actinomycetes have a profound role in the marine environment apart from antibiotic production (Das et al. 2006). The degradation and turnover of various materials are a continuous process mediated by the action of a variety of microorganisms (Jensen et al. 2005a; Lam 2006). There is a speculation that the increase or decrease of a particular enzyme-producing microorganism may indicate the concentration of natural substrate and conditions of the environment (Ramesh and Mathivanan 2009). The cellulolytic activity of marine actinomycetes was described (Chandramohan et al. 1972), chitinolytic actinomycetes were reported by (Pisano et al.1992) and various industrially important enzyme producing actinomycetes have been reported (Ramesh et al. 2009). Actinomycetes are also reported to contribute to the breakdown and recycling of organic compounds (Goodfellow et al . 1984). In addition, they play a significant role in mineralization of organic matter, immobilization of mineral nutrients, fixation of nitrogen, improvement of physical parameters and environmental protection (Good fellow et al . 1983)

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The Bay of Bengal an unexploited ecosystem .that has as a source of novel and biologically potent marine derived compounds by several researchers. According to (Peela et al.2005) numerous marine actinomycetes have been isolated from the samples collected from Andaman coast in Bay of Bengal and the majority of isolates belong to the genus Streptomyces with antibacterial and antifungal activity. In the southern coast of Bangladesh, a novel strain Nonomuraea has been isolated by the Japanese researchers (Ara et al. 2007). Therefore the present work was focused on the isolation of actinomycetes from the sediment samples of Bay of Bengal and their screening for their ability to produce antimicrobials.

More than 70% of our planet's surface is covered by oceans and life on Earth originated from the sea. In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than that in the tropical rainforests. As marine environmental conditions are extremely different from terrestrial ones, it is summarized that marine Actinomycetes have different characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds(Imada C et al .2007).

Around 23 000 bioactive secondary metabolites produced by microorganisms have been reported and over 10 000 of these compounds are produced by Actinomycetes, representing 45% of all bioactive microbial metabolites discovered (Vimal V et al .2009). Among Actinomycetes, around 7600 compounds are produced by Streptomyces species. Many of these secondary metabolites are potent antibiotics, which has made streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry (Ramesh et al .2009; Jensen PR et al . 2007).

II. MATERIAL AND METHODS

2.1 Collection of samples

12 marine soil samples are collected from Nizampatnam coastal region, Guntur district, Andhra Pradesh. Quarterly from marine environments at a depth of 10-15 cm from soil surface and placed in sterile polythene bags, sealed tightly and brought to the laboratory.



FIG;-Collection of soil sample at Nizampatnam coastal region ,Guntur district ,Andhra Pradesh

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2.2 Pretreatment of sample:-

The soil samples are given a physical treatment. In physical treatment – 1gram soil sample is mixed with sterile 9.0 ml distil water , mix properly and exposed at high temperature of 70⁰C for 15minutes to kill the contaminants .

2.3 Enrichment of soil sample:-

In 250 ml conical flask 5ml of the Pre-treated soil sample was added to the 50 ml of the Actinomycetes Isolation broth medium. The medium was supplemented with Nalidixic acid (50 µg/ml) and Amphotericin B (50µg/ml) concentration. The flask were incubated at 30⁰C for 07 days.

2.4 Isolation of Actinomycetes from marine Soil

The organisms were inoculate in Starch casein agar (SCA) medium with 50% seawater was used for isolation of actinomycetes as described by El-Nakeeb and Küster and Williams (2008)

Composition of Starch Casein Agar Medium.

Soluble Starch	- 10.00 grams
Casein	- 0.30 grams
KNO ₃	- 2.00 grams
MgSO ₄	- 0.05 grams
K ₂ HPO ₄	- 2.00 grams
NaCl	- 2.00 grams
CaCO ₃	- 0.02 grams
1FeSO ₄	- 0.01 grams
Agar	- 18.00 grams
Distil water	- 1000 ml
PH	- 7.0.

2.4 Screening for antimicrobial activity:-

The antimicrobial activity was study preliminarily by cup plate method (Haque et al .,1992) against bacteria fungi. The test organism *E. coli* (ATCC 9837), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 9856), and *Pseudomonas aeruginosa*(ATCC 9027). after preliminary testing of the isolates for their antimicrobial activities the most active isolates was selected for the study.

2.5 Screening for Bioactive Compound Producing Actinomycetes

The isolated strains were screened for the production of bioactive compounds. The isolated actinomycetes strains were inoculated in Yeast extract malt extract agar (ISP₂) medium with 50% seawater. The inoculated flask was kept for incubation at room temperature for a period of 5 days on rotary shaker (120 rpm) at 28°C. After incubation the broth was filtered and the filtrate was used to test antimicrobial activity. The overnight cultures like *E. coli* (ATCC 9837), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 9856), and *Pseudomonas aeruginosa*(ATCC 9027) were used as test organisms. The test organisms were spread uniformly on agar plate and wells were bored on the agar surface; then the wells were filled with the culture filtrate. Then plates were incubated at 37°C for 24 to 48 hrs.

Composition of Yeast extract malt extract agar (ISP₂) Medium:-

Yeast extract	- 4.00 grams
Malt extract	- 10.00 grams
Glucose	- 4.00 grams
Agar	- 20.00 grams
Distilled water	- 1000ml
P.H	- 7.3.

2.6. Morphological and Taxonomical Identification of Isolated Strain

Based on the results of screening, one potential strain was selected for further investigations. The potential isolate (JKCM1) was observed for aerial spore, mycelia color, spore chain morphology, and other microscopic characters. The taxonomic identification of actinomycetes sp. was based on Nonomura's key and Bergey's Manual Finally the strain was identified by 16s DNA sequencing were done Synteny life sciences Pvt Limited. Hyderabad and the gene was stored in GenBank, Accession Number: KY656443.1

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2.6.1 Morphological and Cultural Studies

The color of aerial mycelium, substrate mycelium and soluble pigment when grown on different media were observed and recorded. The macro and micro-morphological features of the colonies and the color determinations of the aerial mycelium, substrate mycelium and soluble pigment were examined after 96 h of incubation. Macro-morphology was noted by the naked eye and by observation with magnifying lens.

Micro-morphology:

To study the aerial mycelium and its sporulation characteristics, the following two methods were used.

1. Direct method

Very thin layer of the respective solidified media in petridishes, were inoculated with 0.05 ml of the spore suspension. This was placed near the edge of the plate to serve as a pool of inoculum. Using a sterile loop, four to five equally spaced streaks were made. A number of plates were inoculated in this manner to facilitate observations on different days. Observations were recorded from next day onwards up to 4 days.

2. Inclined cover slip method (Kawato and Shinobu, 1959; Williams and Davis 1967):

Sterile cover slips were placed at an angle of 45° into solidified agar medium in petridish such that half of the cover slip was in medium. Inoculum was spread along the line where the upper surface of the cover slip meets the medium. After full sporulation, the cover slips were removed and examined directly under the microscope.

2.6.2 Physiological Characteristics:

1. Gram - staining .

To study the Gram's reaction of the culture, a 48 h culture was used. The experimental protocol as described by Salle (1948) was followed

2. H₂S production (Shirling and Gottlieb, 1966):

Many organisms, in their metabolism of sulphur containing organic compounds, liberate hydrogen sulphide in considerable amounts. This can be demonstrated if sulphide producing cultures are grown on media containing salts of iron resulting in the formation of a black or bluish black precipitate. The use of H₂S production as a taxonomic implement was suggested by many authors. The inoculated peptone-yeast extract-iron agar (ISP-6) slants were incubated at 28°C for 5 days. Observations for the presence of characteristic greenish brown, brown, blackish brown, bluish black or black colour of the substrate, indicative of H₂S production, were made every 24 h upto 5 days.

3. Gelatin hydrolysis (Gordon and Mihm, 1957):

This represents the ability of microorganisms to hydrolyze or liquefy gelatin. Most of the species of Streptomyces bring about liquefaction of gelatin but the rapidity of liquefaction varies with the species. The non-pigmented forms are most active while the pigmented forms are least active. For this test, the isolates were grown on gelatin agar plates for two days at 55°C. At the end of incubation period, the plates were flooded with 1 ml of the following solution.

Mercuric chloride : 15 g
Conc. HCl : 20 ml
Distilled water : 100 ml

The extent of hydrolysis was noted by comparing the width of the clear zone around the growth. The widths of the hydrolyzed zone and growth zone were measured and the ratio of hydrolyzed zone and growth zone was calculated.

4. Casein hydrolysis (Salle, 1948):

The proteolytic activity was studied with milk-casein agar medium by measuring the hydrolyzed zones after incubating the inoculated plates at 28°C for 48 h. The extra-cellular protease (caseinase) activity of the isolates was determined qualitatively as the ratio of the diameter of the hydrolyzed zone and that of the growth on the milk-casein agar medium.

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5. Starch hydrolysis (Salle, 1948):

Numerous actinomycetes are able to hydrolyze starch rapidly by the action of amylolytic enzymes. For this test, the selected isolates were grown for 5 days on starch agar plates. At the end of incubation period, the plates were flooded with weak iodine solution. The width of hydrolyzed zone around the growth versus the width of the growth was measured.

Composition of iodine solution:

Potassium iodide : 3g

Iodine : 1g

Distilled water : 100mL.

6. Nitrate reduction test (Salle, 1948):

The reduction of nitrate to nitrite has been universally used among the criteria for species differentiation. The reduction is the result of the use of NaNO₃ or KNO₃ as an electron acceptor with some organisms. Nitrate (NO₃) and NO₂ serve as sources of nitrogen for the synthesis of organic nitrogen compounds or they may function as H⁺ acceptors in reactions concerned with the organisms energy metabolism. The first step in the reduction of NO₃ involves its conversion to NO₂ by an enzyme system, which is adaptive in nature and is known as nitratase. 5ml of nitrate broth medium was inoculated with a loopful of spores and incubated at 28°C for 5 days. Controls were also run without inoculation. After 5 days, the clear broth was tested for the presence of nitrite in the following way.

Reagents:

a) Alpha -Naphthylamine test solution:

Alpha-Naphthylamine : 5.0g

Conc. H₂SO₄ : 8.0 ml

Distilled water : 1 L

To the diluted sulphuric acid,

Alpha naphthylamine was added and stirred until solution was effected.

b) Sulphanilic acid test solution:

Sulphanilic acid : 8.0 g

Conc. H₂SO₄ : 48 ml

Distilled water : 1L

Sulfuric acid was added to 500 ml of water. Then sulphanilic acid was added followed by water to make up the volume.

Procedure:

To 1 ml of the broth under examination and 1 ml of control, two drops of sulphanilic acid solution followed by two drops of a-naphthylamine solution were added. The presence of nitrite was indicated by a pink, red or orange colour and absence of colour change was considered as nitrite negative. In the later case presence or absence of nitrate in the broth under examination was confirmed by adding a pinch of zinc dust, after the addition of the reagents, when the unreduced nitrate, if present, gave a pink, red or orange colour.

7. Growth temperature (Williams et al. 1989):

Ability of the isolates to grow at different temperatures was studied at 15° C, 25°C, 37°C and 40°C. The selected isolates were inoculated on starch casein agar medium and jowar starch medium slants and incubated at the different temperatures as mentioned above. Results were recorded on 3rd day and 5th day.

8. Chemical tolerance pH(Williams et al, 1989):

10 ml quantities of glycerol-nutrient broth with pH levels of 5.2, 8.0, 9.0, and 10.0 were inoculated and incubated for 3 to 5 days. Then the tubes were examined for the extent of growth of the organism

2.7. Production and Extraction of Bioactive Compounds

All the media used in this study were prepared in 50 mL filtered seawater and 50 mL distilled water. Hence the growth was optimum at that proportion. Spores of potential actinomycete strain were scrapped from starch casein agar and inoculated into 50 mL of inoculation medium in 250 mL conical flask and kept in rotary shaker at 120 rpm for 48 hours at 28°C. Then 10% of inoculum was transferred into 100 mL of production medium and kept in rotary shaker

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at 120 rpm for 7 days at 28°C. After fermentation, mycelium and supernatant were separated first by filtration and finally by centrifugation at 10,000 rpm for 30 minutes at 4°C. The extracellular compounds from culture supernatant were extracted by liquid-liquid extraction method using equal amount of ethyl acetate and concentrated by Rota evaporation.

III. RESULTS AND DISCUSSION

3.1. Isolation and Screening of Actinomycetes

Two morphologically different strains along with clear zones around the colony were observed in the starch casein medium after 5 days of incubation (Figure 1). The strain of JKC-M1 was screened for the production of bioactive compounds; the strain exhibited good antimicrobial activity against different pathogenic bacteria as shown in Table 1. The strain showed maximum activity was selected for morphological, cultural, molecular characterization and bioactive compound production. These results were correlated with the bioactive compounds from *N. pseudo brasiliensis* and *N. mediterranei*



Fig:-Actinomycetes isolation agar plate method showing growth of actinomycetes colonies

3.4 Screening for antimicrobial activity:-

The screening for antimicrobial activity revealed that 04 out of 25 isolate marine actinomycetes ,after only 01(JKC-M1) was given highly activity shown in figure 4.2 and table 4.1

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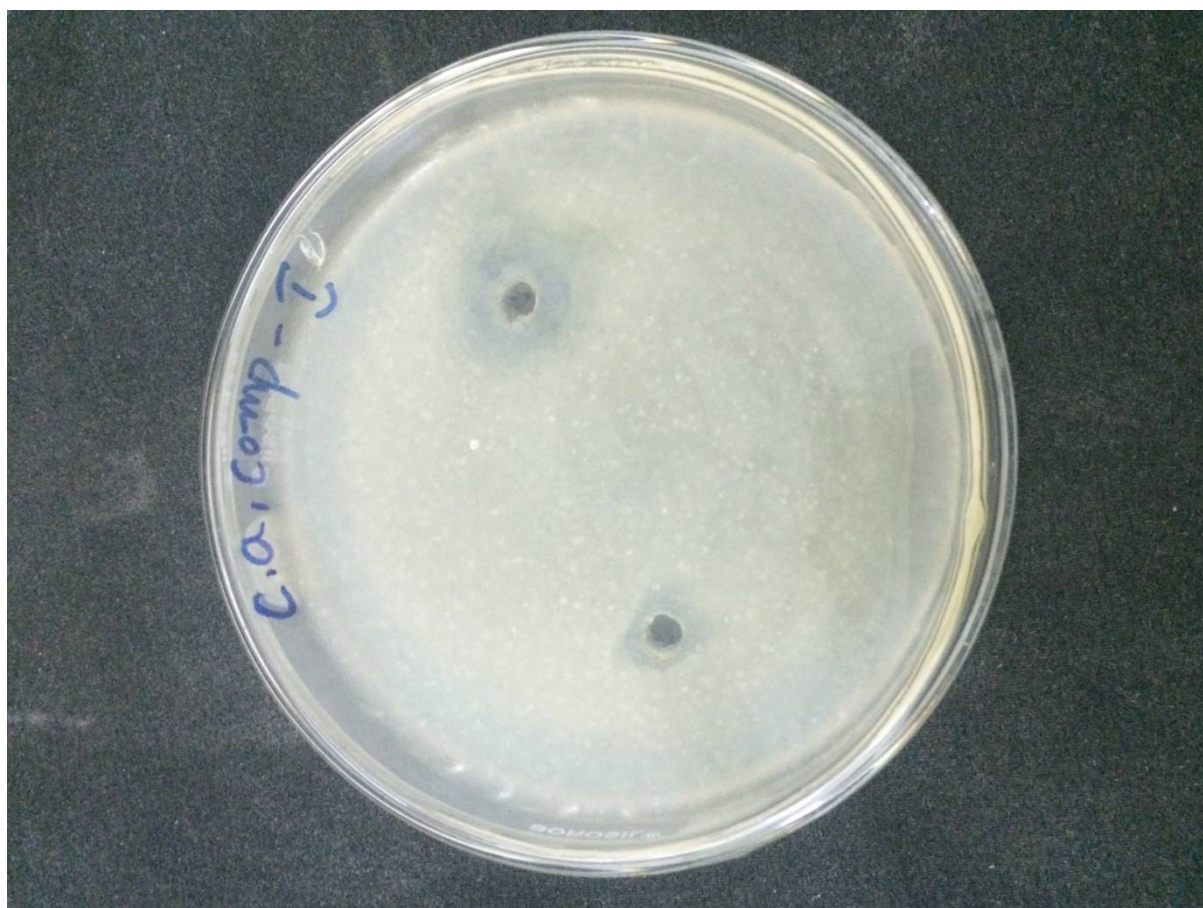


Fig:- Antimicrobial activity of marine actinomycetes (JKC-M1)

SNO	TEST SAMPLE	TEST ORGANISM	NAME OF THE MEDIA	DIAMETER (in cm)
1	JKC M1	<i>E.coli</i>	SCA	0.3
2	JKC M1	<i>B.megaterium</i>	SCA	0.5
3	JKC M1	<i>B.cereus</i>	SCA	2.0
4	JKC M1	<i>C.albicans</i>	SCA	0.5
5	JKC M1	<i>S.aureus</i>	SCA	0.5
6	JKC M1	<i>X.campestris</i>	SCA	0.5

Table :- 1: Isolated colonies of actinomycetes showing clear zone on Starch casein agar medium.

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S.NO	TEST SAMPLE	TEST ORGANISM	NAME OF THE MEDIA	DIAMETER (in cm)
1	JKC M1	<i>E.coli</i>	ISP2	1.1
2	JKC M1	<i>B.megaterium</i>	ISP2	1.5
3	JKC M1	<i>B.cereus</i>	ISP2	2.2
4	JKC M1	<i>C.albicans</i>	ISP2	1.5
5	JKC M1	<i>S.aureus</i>	ISP2	2.0
6	JKC M1	<i>X.campestris</i>	ISP2	1.5

Table 2: Isolated colonies of actinomycetes showing clear zone on Yeast extract malt extract agar ISP2 medium.

3.2. Morphological and Biochemical Test

Micro- and macroscopic characteristics: the aerial mycelium formed monopodially branched spore-bearing hyphae with the shape of loops, open or compact spirals with 3–6 curves. The strain was clearly polymorph and the colonies were completely covered by aerial mycelium and it formed a clear zone around the colony. These morphological characters are closely agreed with the findings of Good fellow and Hoshino et al.. In carbon assimilation test the isolate JKC-M1 grew poorly in the presence of sucrose and fructose as a sole carbon source and formed abundant mycelium on the media with glucose and maltose, whereas in nitrogen utilization test, it has grown abundantly on glutamic acid and poorly on histidine, methionine, and leucine. It also showed other biochemical characters like starch, casein, and gelatin hydrolysis as shown in Table 4.3

Characters	JKC M1
Colony appearance	Mycelial (cottony)
Sporulation of aerial mycelia	Long chains
Colony colour	White
Gram's staining	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
H ₂ s production	+
Nitrate reduction	-
PH	8 TO 9
Temperature	30 ^o c

Table 2: Macroscopic and microscopic tests of JKCM1 strain

3.3. Identification of Actinomycetes

The taxonomic identification of the JKC M1 was based on 16s rDNA analysis. The 16s rDNA sequence of the strain was compared with the sequences in GenBank using BLAST and aligned with the sequences retrieved from NCBI GenBank database using the Clustal method. The phylogenetic tree was constructed based on neighbor joining tree method and illustrated in Figure 3. The database was deposited in NCBI GenBank k, Accession Number: KY656443.1. Based on the cultural, morphological, physiological, and molecular analysis, the JKC M1 was identified as *Nocardopsis alba*.

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SEQUENCES :-

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GGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGA
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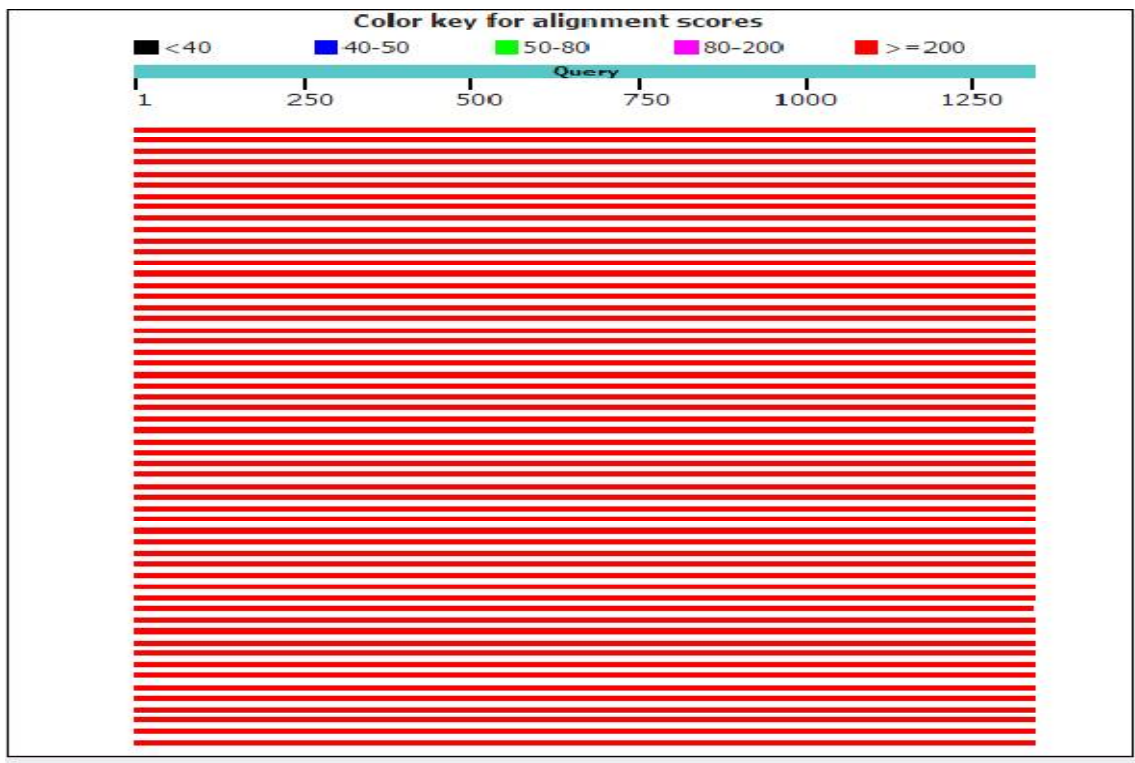


FIG:- BLAST DATA: (Alignment view using combination of NCBI)

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Streptomyces sp. strain MUM212 16S ribosomal RNA gene, partial sequence	2447	2447	100%	0.0	100%	KY865443.1
Streptomyces sp. S1-SC17 16S ribosomal RNA gene, partial sequence	2388	2388	100%	0.0	99%	KM677394.1
Streptomyces sp. strain MUM265 16S ribosomal RNA gene, partial sequence	2385	2385	100%	0.0	99%	KY865444.1
Streptomyces sp. DMU-NKS-7 16S ribosomal RNA gene, partial sequence	2385	2385	100%	0.0	99%	KF746335.1
Streptomyces missouriensis strain NIIST A2 16S ribosomal RNA gene, partial sequence	2383	2383	100%	0.0	99%	KJ685376.1
Streptomyces sp. Mem06 16S ribosomal RNA gene, partial sequence	2383	2383	100%	0.0	99%	KP083458.1
Streptomyces hygroscopicus strain FoRh25 16S ribosomal RNA gene, partial sequence	2383	2383	100%	0.0	99%	KM370343.1
Streptomyces sp. R20-5 gene for 16S ribosomal RNA, partial sequence	2383	2383	100%	0.0	99%	AB841057.1
Streptomyces sp. R14-5 gene for 16S ribosomal RNA, partial sequence	2383	2383	100%	0.0	99%	AB841042.1
Streptomyces sp. R9-4 gene for 16S ribosomal RNA, partial sequence	2383	2383	100%	0.0	99%	AB841027.1
Streptomyces hygroscopicus subsp. hygroscopicus gene for 16S rRNA, partial sequence, strain: NBRC 3401	2383	2383	100%	0.0	99%	AB184750.1
Streptomyces ovesensis gene for 16S rRNA, partial sequence, strain: NERC 13302	2383	2383	100%	0.0	99%	AB184515.1
Streptomyces sp. MJC249T 16S ribosomal RNA gene, partial sequence	2381	2381	100%	0.0	99%	KF682210.1
Streptomyces sp. MJC246T 16S ribosomal RNA gene, partial sequence	2379	2379	100%	0.0	99%	KF682208.1
Streptomyces missouriensis strain RB45 16S ribosomal RNA gene, partial sequence	2377	2377	100%	0.0	99%	KY563723.1

FIG: Sequence Producing Significant Alignments

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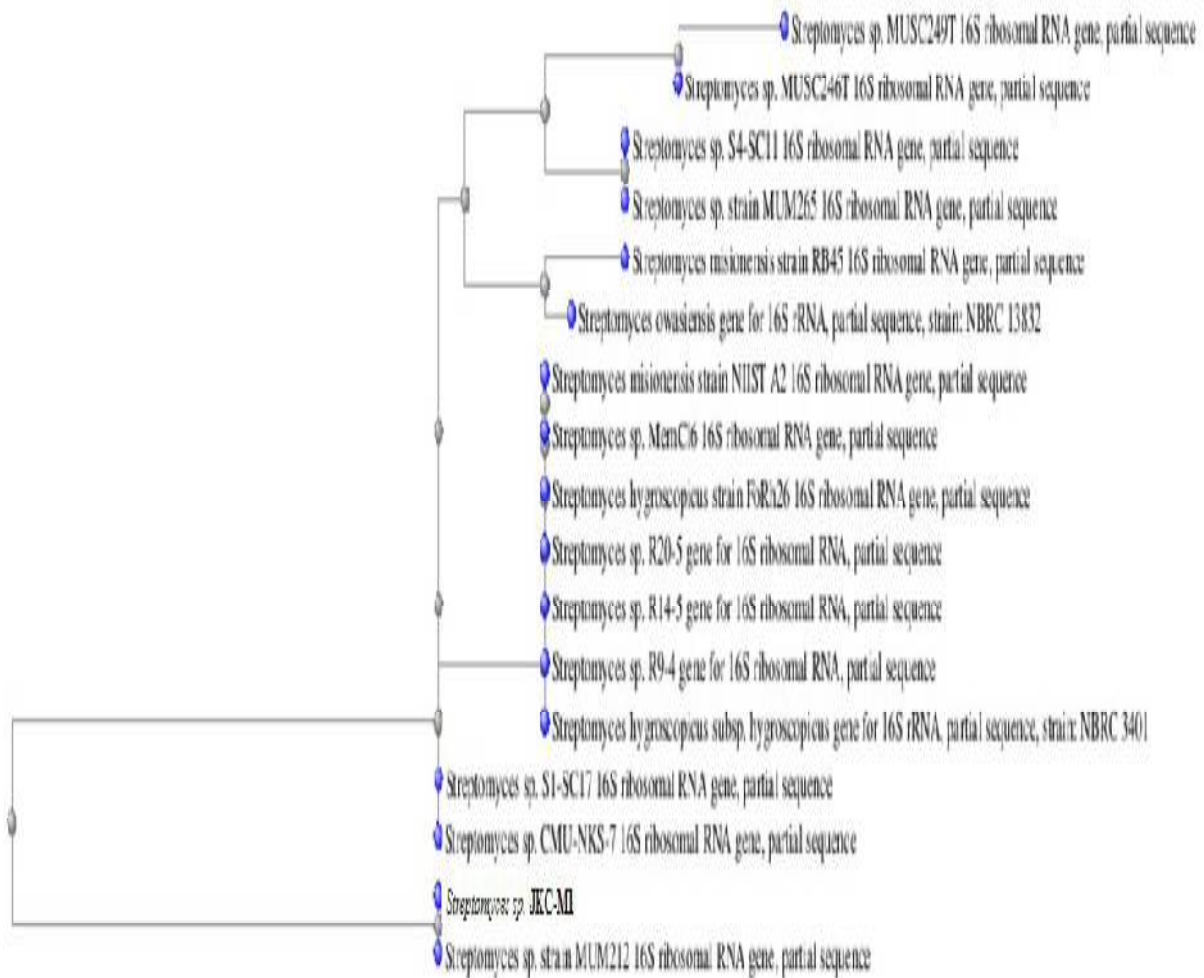


Figure 3: Phylogenetic tree of JKC M1

IV. SUMMARY

In present study, 23 isolates of actinomycetes were isolated from marine soil and water that randomly collected Nizampatnam, Guntur district, Andhra Pradesh, India. Actinomycetes isolates have more potential in antimicrobial activity the antimicrobial reaction showed high inhibition zone formation. The isolates of actinomycetes were identified as *Streptomyces* sp. JKC-M1. Majority of actinomycetes are free-living saprophytic bacteria found widely distributed in marine soil, water and colonizing plants. The population of actinomycetes has identified as one of the major groups of soil population, which may vary with the soil type. Actinomycetes from soil are interesting isolates which show the potential to produce bioactive compounds that may be useful for human cancer colon treatment and anti-diabetic treatment.

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