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Production and Purification of Novel Antibiotics from Marine Actinomycetes

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Abstract: **Fifteen marine and water samples were collected from different places of east cost of India, such as Kakinada, Chennai, Mahabalipuram, Kovalam, Vishakhapatnam and Kumbakonam. On screening the samples one potential actinomycetes was obtained. Colony morphology, Gram staining and biochemical tests revealed the organism as *Streptomyces* spp. Antimicrobial compound released from *Streptomyces* spp. were tested against one Gram positive organism, *Staphylococcus aureus* ATCC-259233 and one Gram negative organism, *Escherichia coli* FPFC-1407. Optimization of physico chemical parameters like incubation period, temperature, pH, different carbon sources, nitrogen sources, varied concentration of salinity was performed for antibiotic production by *Streptomyces* spp. In the current study, strain improvement using random physical (U. V. irradiation) and chemical (EtBr) mutations were also carried out. The antibiotic production by *Streptomyces* spp. was increased after optimization.**

Key words: **Antibiotics, Novel, Actinomyces, Marine, production and purification.**

I. Introduction:

Microbes naturally produce defensive chemicals as a way of competing with other species for limited resources and survive these are known collectively as antimicrobials. Most of the current antibiotics used to treat infections in humans and animals are derived from microbes. Antibiotic resistance is a feature of bacterial evolution. Bacteria can become resistant either by receiving genes from other species of already resistant bacteria or through spontaneous mutations or changes in their genetic code (Simon *et al.*, 2013).

Actinomycetes a significant component of microbial organisms in both terrestrial and marine body like marine water, marine sediments (You *et al.*, 2005). Present antibiotics are not up to the mark to defend with pathogenic organisms. It leads to identify new strains from other sources like Marine bodies. Recent studies have also shown that Actinobacteria can be isolated from mangrove swamps, other coastal environments, and even deep ocean sediments (Tae *et al* ., 2005). Though the deep sea area is geographically vast, scientific knowledge and research on deep sea microbial diversity is meager (Das *et al.* 2006). However, it has been shown to be a good source of novel microorganisms for the discovery of new antibiotics (Bull *et al*. 2000). The Actinomycetes are active components of marine microbial communities (Jensen *et al*., 2005) and form stable, persistent populations in various marine ecosystems. The discovery of several new marine Actinomycete taxa with unique metabolic activity in their natural environments (Fenical *et al.*, 2006), and their ability to form stable populations in different habitats and produce novel compounds with various biological activities

Current study, we attempted to describe the isolation of Actinomycetes populations from different places of coastal regions of Tamil Nadu and Andhra Pradesh and production of secondary metabolites as to screen the antagonistic Actinomycetes. Strains were characterized based on morphological, biochemical, cultural and physiological

characteristics. And purification of antibiotic is to identify its novel characteristics of compound.

II. Materials and Methods:

2.1 Collection of Marine Samples:

Fifteen marine water and soil samples were collected from different places of east cost of India, such as Kakinada, Chennai, Mahabalipuram, Kovalam, Vishakhapatnam and Kumbakonam. Soil samples were collected from 8-10 cm depth and taken to the laboratory in sterile polythene bags. Water samples were collected 100 to 200 meter away from cost in a sterile plastic bottle.

2.2 Isolation of Pure Culture:

Actinomycetes were isolated by serial dilution method using Starch-Casein-agar media (SC-agar). 1 gm of Marine samples was taken into 10 ml of saline sample. 1 ml of marine water sample taken into 9 ml of 5% saline sample and serially diluted, the organisms were isolated using pour plate method. Plates incubated at 37^{o}c and monitored for seven days. After incubation white precipitated colony observed and maintained on SC-agar slants.

2.3 Screening of Antimicrobial Activity of Pure Isolates:

Preliminary screening for antimicrobial activity of organisms was done by using Antibiogram technique. 1 inch of watt man filter paper placed in Petri dish and SC-agar media poured on it. Isolates are spread evenly on media and incubated for 3 days. Slice of agar media along with whatt man filter paper cut and placed inverted position on *E.coli* spread nutrient media plates and Incubated for the period of 24 hours and measured the zone of inhibition.

2.4 Test organisms:

Two test organisms were used to test antimicrobial activity of isolates are one Gram-positive *Staphylococcus aureus* ATCC-259233 and Gram-negative *Escherichia coli* FPFC-1407. They were maintained on nutrient agar slants at 4^{o}c.

2.5 Optimization for production of antibiotic:

2.5.1 Optimization of incubation period for production of antibiotic

Two hundred ml of SC-Broth was used for the production of antibiotic and incubated for seven days. Alternative day the activity was observed by using well diffusion method by Muller and Hilton Agar media. Tetracycline was used as the control for the test. *Streptomyces spp*. inducing maximum zone of inhibition was selected and used for production of antibiotics and for next objective.

2.5.2 Optimization of temperature for production of antibiotic:

The isolate, *Streptomyces spp*. inoculated in SC-broth was incubated at different incubation temperatures (20^{0}C, 37^{0}C, 45^{0}C and 55^{0}C). After 92 hours of incubation, antimicrobial activity was conducted against the test organisms.

2.5.3 Optimization of pH for production of antibiotic:

Optimum pH was studied by varying the SC-broth pH (4, 5, 6, 7 and 8) and *Streptomyces spp* was inoculated. The broths were incubated for 92 hrs at 45^{0}C and were further tested for antimicrobial activity against the test organism.

2.5.4 Optimization of carbon sources for production of antibiotic:

Different carbon sources (cellulose, maltose, dextrose, lactose, fructose and xylose) was supplemented to SC-broth (pH 5) and inoculated with *Streptomyces spp.* The media was incubated at 45^{0}C for 92 hrs. Antimicrobial activity was evaluated against the test organisms.

2.5.5 Optimization of nitrogen sources for production of antibiotic:

Streptomyces spp. was inoculated in SC-broth (pH 5) with different nitrogen sources and incubated at 45^{0}C for 92 hrs. The broth was examined for antimicrobial activity against the test organism.

2.6 Effect of mutation on antibacterial activity:

The isolates showing efficient antibacterial activity were further selected to study the effect of mutation on antibiotic activity.

2.6.1 Physical Mutation:

Selected isolates were cultured in SC-Broth for ninety six hours at 37^{o}C. LB-agar plates were prepared and the plates were inoculated with l00 µl of sample and were exposed to UV irradiation at a distance of 30cm for 10min and incubated for twenty four hours at 37^{o}C. The colonies from the UV exposed plates were cultured in SC-Broth for incubation at 45^{o}C for ninety six hours, broth was centrifuged at 10000 rpm for 10 min and supernatant was collected to examine post mutation Antimicrobial activity.

2.6.2 Chemical Mutation:

Selected isolate was inoculated in LB-Broth and incubated for twenty four hours at 37^{o}C with different concentration of Ethidium Bromide. After incubation period, 100 µl of sample was inoculated in SC-Broth and kept for incubation for 96 hours at 45^{o}C. Supernatant was collected after centrifugation at 1000 rpm for 10 min and was observed for post mutational antibacterial activity.

Test organisms *Escherichia coli* and *Staphylococcus aureus* are grown on Nutrient Broth. After incubation of the test organisms they were spread plate on Nutrient agar plates. Antibacterial activity was evaluated by well diffusion method and diameter of each zone was recorded by using millimeter scale.

III. RESULTS AND DISCUSSION:

3.1 Isolation and Screening of pure culture:

Several colonies of different morphology were obtained from fifteen marine water and soil samples and a total of two Isolates were selected based on the distinct colony morphology, Gram positive character and microscopic characters. All the selected isolates were screened for the highest antimicrobial activity and the actinomycetes with a high antimicrobial activity were maintained on SC-agar slants for further studies. The isolate selected was characterized as *Streptomyces* spp. based on colony morphology, Gram character and biochemical tests.

3.2 Optimization for production of antibiotic:

A large number of factors influence the growth and production of bioactive metabolites by actinomycetes. Parameters like fermentation time, temperature, initial pH etc have profound effect on production of bioactive metabolites. Together with these parameters, the combination of media components also influences growth and metabolite production (Song *et al*., 2012; Khan and Tripathi, 2011)

3.2.1 Optimization of incubation period for production of antibiotic

Period of incubation is an important factor to affect the fermentation (Song *et al.*, 2012). *Streptomyces* spp. exhibited antibacterial activity against *S aureus* ATCC-259233 and *E coli* FPFC-1407 and is recorded as shown in fig 1. *Streptomyces* spp. highest activity was on the fourth day, zone of inhibition against *S aureus* ATCC-259233 was 13mm and against *E coli* FPFC-1407 was 8mm. Highest level of antimicrobial metabolite was produced by *Streptomyces* spp against *Bacillus subtilis* after ten days of incubation period (Ripa *et al*., 2009). In a study, Saha *et al*.20 observed production of antimicrobial metabolite production by *Streptomyces* sp. MNK7 on 3rd day which reached maximum level after 10 days of incubation and later declined. The isolate *Streptomyces* species SRDP-TK-07 produced bioactive metabolites to higher extent on 7th day of incubation (Rakesh *et al*., 2014).

3.2.2 Optimization of temperature for production of antibiotic:

Incubation temperature had an effect on the production of antibiotic, the production increased with increase in

temperature with a maximum at 45˚C and declined beyond the temperature. From the Fig 2, *Streptomyces* spp. exhibited maximum inhibition of 14mm against *S aureus* ATCC-259233 and 12mm *E coli* FPFC-140. *Streptomyces albidoflavus* biomass and antibiotic production levels were maximum at 35˚C (Narayana and Vijayalakshmi, 2008). Kumari *et al*., 2013 reported 35˚C as optimum temperature for antibiotic production by *Streptomyces* sp. US7 MTCC 8723.

3.2.3 Optimization of pH for production of antibiotic:

The role of pH in the antibiotic production by *Streptomyces spp* has been presented in the Fig 3. Antibiotic was produced in a range of pH (4, 5, 6, 7 and 8) and the activity was exhibited high in acidic pH of 5 for *E coli* FPFC-1407 while 6 for *S aureus* ATCC-259233. Zone of inhibition of 13mm and 9mm was recorded against *E coli* FPFC-1407 and *S aureus* ATCC-259233, respectively. In a study conducted by Uddin *et al.,* 2013 optimum pH of the medium was adjusted to basic scale of 9 for high activity of *Streptomyces albolongus* against *S. aureus* (zone of inhibiton of 33mm). The optimal pH was 7.0 for Actinomycetes YJ1 against *Sclerotinia sclerotiorum* (Song *et al.,* 2012)

3.2.4 Optimization of carbon sources for production of antibiotic:

The nutritional source like carbon, nitrogen and minerals as well as the environmental factors are known to have profound effect on antibiotic production by Actinomycetes (Himabindu and Jetty, 2006). The production media supplemented with Lactose as sole source of carbon exhibited highest activity against *E coli* FPFC-1407 with a zone of inhibition of 23mm and 24mm against *S aureus* ATCC-259233(Fig 4.). The media supplemented with other carbon sources (Cellulose, Maltose, Dextrose, Fructose and Xylose) had an effect on the antimicrobial activity of *Streptomyces spp* against the test organism with a marginal decrease in activity when compared to Lactose. For the production of antimicrobial metabolite by *Streptomyces sp*. RUPA-08PR the optimum carbon source was 2% glucose, with maximum zone of inhibition against *Bacillus subtilis* (Ripa *et al*., 2009). A report by Narayana and Vijayalakshmi, 2008 recorded maltose as the optimum sole source of carbon for *Streptomyces albidoflavus* to exhibit high levels of biomass and antimicrobial activity against *Bacillus subtilis, Pseudomonas aeruginosa and Fusarium udum* (zone of inhibition of 17mm, 18mm and 21mm respectively was observed).

3.2.5 Optimization of nitrogen sources for production of antibiotic:

The medium supplemented with different nitrogen sources, both organic and inorganic, exhibited varied range of antimicrobial activity by *Streptomyces spp* against the test organsisms (Fig 5). Inorganic source, sodium nitrate, exhibited maximum zone of inhibition of 32mm and 29mm against *E coli* FPFC-1407 and *S aureus* ATCC-259233, respectively. Soybean meal exhibited highest antimicrobial against *Bacillus subtilis, Pseudomonas aeruginosa, Fusarium udum* with 23mm, 24mm, 26mm zone of inhibition (Narayana and Vijayalakshmi, 2008). Highest activity was exhibited by *Streptomyces sp*. RUPA-08PR in a medium supplemented with organic compound, yeast, against *B subtilis* (Ripa *et al*., 2009).

3.3 Effect of mutation on antibacterial activity:

3.3.1 Physical Mutation:

Phillips (1960) reported that UV-mutated actinomycetes increase antibiotics production than that of non-mutated actinomycetes. The mutants didn't lose the property of producing antibiotics, the activity of the metabolite produced by *Streptomyces* spp exhibited maximum zone of inhibition of 11mm against *E coli* FPFC-1407 at ten minutes exposure to U. V. and 9mm of zone against *S aureus* ATCC-

259233 for five and ten minutes exposure to U. V. A minimal decrease in the activity was noted with different exposure to U. V. radiations (Fig 6). U. V mutated *Actinomadura livida*

showed inhibition zone of 14mm against *K. pneumoniae* (Ashok et al., 2014)

3.3.2 Chemical Mutation:

Data on the effect of different concentrations of EtBr mutagen is presented in the Fig 7. The antimicrobial substance produced by the mutant *Streptomyces* spp was recorded high with 2µL EtBr volume exhibiting 1mm and 10mm Zone of inhibition against *E coli* FPFC-1407 and *S aureus* ATCC-259233 respectively.

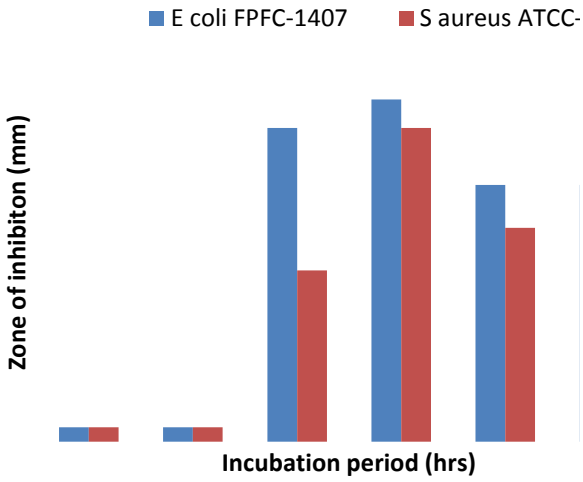

Fig 1. Optimizing the incubation period for antibiotic production by *Streptomyces* spp.

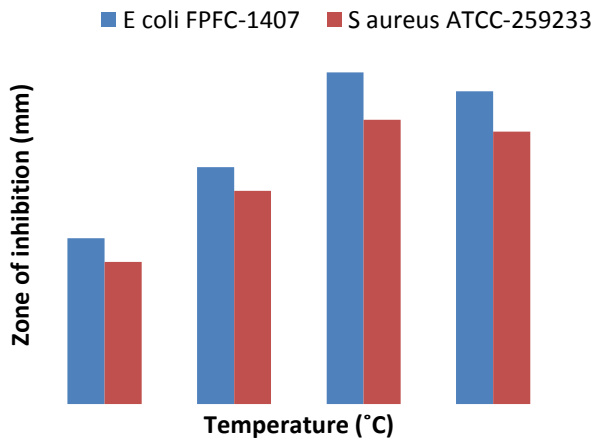

Fig 2. Effect of temperature on antibiotic production by *Streptomyces* spp.

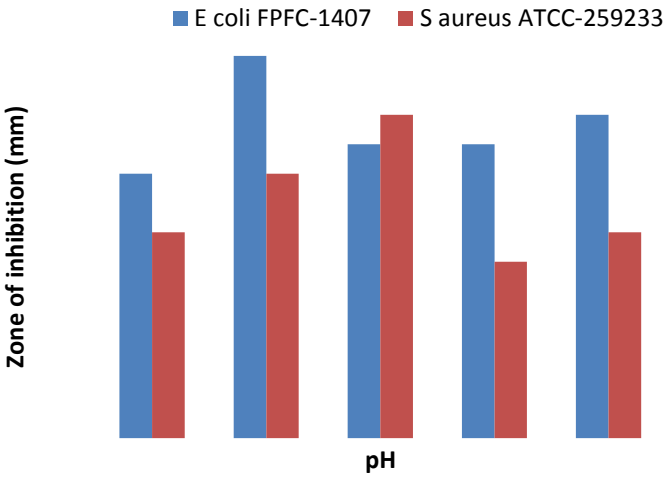

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Fig 3. Influence of pH on antibiotic production by *Streptomyces* spp.

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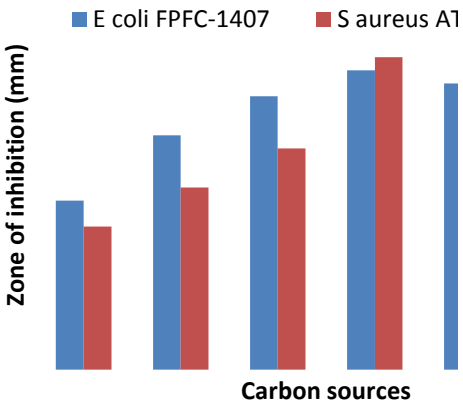

Fig 4. Effect of carbon sources on antibiotic production by *Streptomyces* spp.

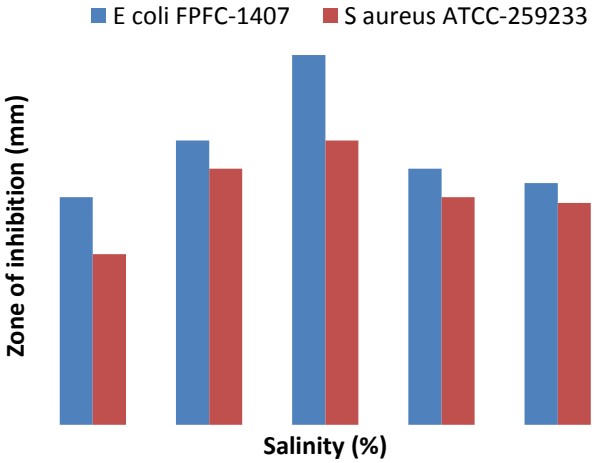

Fig 6. Effect of salinity sources on antibiotic production by *Streptomyces* spp.

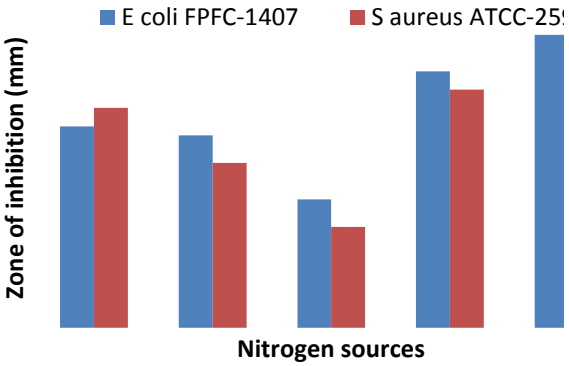

Fig 5. Effect of nitrogen sources on antibiotic production by *Streptomyces* spp.

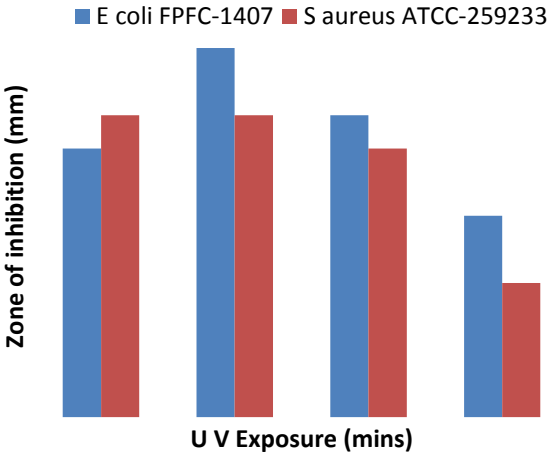

Fig 7. Effect of U. V. exposure on antibiotic production by *Streptomyces* spp.

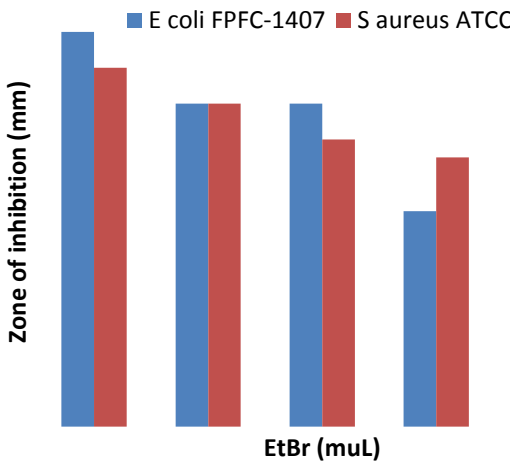

Fig 8. Effect of EtBr on antibiotic production by *Streptomyces* spp.

Conclusion: The current study was aimed to optimize the antibiotic production by *Streptomyces* spp. From the data obtained, *Streptomyces* spp was able to produce antibiotic in wide range of incubation temperature, pH, carbon sources, nitrogen sources, salinity. Optimum incubation period and temperature for the production of antibiotic by *Streptomyces* spp. was 92hrs and 45˚C respectively. Highest zone of inhibition was recorded as 13mm and 9mm against *E coli* FPFC-1407 and *S aureus* ATCC-259233 respectively in the medium adjusted to pH 5. Up to 23 mm and 24mm of zone of inhibition was exhibited in the medium supplemented with lactose by *Streptomyces* spp. Zone of inhibition was as wide as 32mm and 29mm against *E coli* FPFC-1407 and *S aureus* ATCC-259233 respectively in a medium supplemented with Sodium nitrate. 2.5% of salinity was optimum for *Streptomyces* spp for antibiotic production. On subjecting the cultures to mutation, the mutants were able to produce the antibiotic and the zone of inhibition was high with ten minutes exposure to U.V and 2µL of EtBr. This study might be used for an alternative for antibiotics used presently against pathogenic infections caused by *E coli and S aureus* spp.

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