S.Kannan et al /J. Pharm. Sci. & Res. Vol. 11(10), 2019, 3481-3484

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Screening and Characterization of Antimicrobial Compound Produced From Selected Marine Actinomycte

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Abstract:

In the present study, totally four strains of actinomycetes were isolated from marine soil in Bay of Bengal, near the Chennai of Tamilnadu in India. *Streptomyces species* was identified as potential antibiotic producer with antimicrobial activity against Gram-positive and Gram- negative species. Starch casein was shown to be the best growth medium as well as suitable substrate for antibiotic production. In the present study chalky white coloured and gray coloured isolated were noted. The production of citrase and oxidase are considered for characterizing Streptomyces. The present investigation revealed that *Streptomyces species* could produce detectable quantities of antimicrobial compounds in SCA medium. The antimicrobial compound extracted by using ethyl acetate were purified and separated by column and thin layer chromatography and was further characterized using UVspectra studies. The present study showed that the antibacterial efficacy of *Streptomyces* species was maximum against *Staphylococcus aureus* (38±0.44 mm) followed by *Bacillus subtilis* (29±0.36 mm), *Escherichia coli* (25±0.28 mm) and *Klebsiella pneumoniae* (11±0.24 mm).These results confirmed the presence of immense potential in screening and characterization of new compounds for therapeutic purposes. Thus, the present study was undertaken to identify new drugs from marine microorganisms against human pathogens.

Key words: Marine soil, *Streptomyces species*, Antibacterial activity and Human pathogens.

INTRODUCTION

The discovery of novel antibiotic and non-antibiotic compounds lead molecules of pharmaceutical interest through microbial secondary metabolite screening is becoming increasingly fruitful. There is wide acceptance that microorganisms are virtually unlimited sources of novel substances with many therapeutic applications. Among the microorganisms, actinomycetes gain special importance, as they are the most potent source for the production of antibiotics and other bioactive secondary metabolites *Streptomyces* is best recognized genus of actinomycetes. A marine microorganism has produced novel metabolites that ensure their survival in extreme habitats and also offers the potential for production of bioactive metabolites not observed in terrestrial microorganisms (Fenical *et al.*, 1999). Marine actinomycetes are efficient producers of new secondary metabolites that show a range of biological activities including antibacterial, antifungal, anticancer and insecticidal and enzyme inhibition. In the present work, antagonistic actinomycete strain was isolated and their bioactive novel compound was partially purified and characterized. To understands the actinomycetes population in Bay of Bengal, Chennai of Tamilnadu and to find out the potentiality of the production of antimicrobial compounds..

MATERIALS AND METHODS

Sea shore soil sample collection

The marine sea shore soil samples were collected from at the depth of 20cm, 20 meter near the sea side in Bay of Bengal, near the Chennai of Tamilnadu during the period of February 2019.

Isolation of actinomycetes

Starch casein agar medium was prepared and sterilized at 121°C in 15 lbs pressure for 15 min. Then it was supplemented with Amphotericin B (50 µg/l) and Tetracycline (20 µg/l) to prevent the bacterial and fungal growth. The medium was poured into the sterile Petri plates. 1g of marine sea shore soil sample was suspended in 9 ml of sterile double distilled water. Then the samples were serially diluted for up to 10^{-6} and 0.1 of the diluted samples was spread over the agar plates in triplets. The inoculated plates were incubated at 28±2°C for seven to ten days. After incubation, the actinomycetes were observed, purified using subculture method and maintained in starch casein agar medium for further investigation. For actinomycetes culture, Starch casein broth and Nutrient broth were used.

Identification of actinomycetes colonies

Morphological characterization

Morphological characterization was performed with a magnified lens on actinomycete strains grown for 3 to 14 days on starch casein agar plate. Colony morphology was recorded with respect to aerial colour, aerial mycelium, size, nature of colony, reverse side colour and pigmentation and the isolates were observed under the microscope and also performed the gram staining and acid fast staining.

Biochemical characterization

Biochemical characterization was performed with a actinomycete strains grown for 3 to 14 days on starch casein agar plate. Indole test, Methyl red test, Vogesproskauer test, Citrate utilization test, Catalase test, Oxidase test, Starch hydrolysis and Casein Hydrolysis test was performed.

Organisms tested for biological characterization

Bacterial cultures were obtained from Microbiological Laboratory, Erode. Gram positive bacteria's like *Staphylococcus aureus* and *Bacillus subtilis* and gram negative bacteria's like *Escherichia coli* and *Klebsiella pneumoniae* were used in biological characterization.

Screening for biological characterization

Primary screening

Antimicrobial producing property of the actinomycetes was screened by cross streak method (Egorov, 1985). In this single streak of the actinomycetes was made on the surface of the modified Bennett's agar medium and incubated at 28±2°C. After observing a good ribbon like growth of the actinomycetes on the petri plates, the pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Klebisella pneumoniae*) were streaked at right angles to the original streak of actinomycetes and incubated at 37°C. The inhibition zone was measured after 24 and 48 hrs.

Secondary screening

Production of bioactive metabolites

Starch casein agar media used for the growth as well as high production of bioactive secondary metabolites for the selected microbial strains. Fermentation was set in one liter Erlenmeyer flasks containing 500 ml culture media. A loopful of culture from a pure colony was used as inoculums. The cultures were incubated at 28°C with 180 rpm agitation for 15 days. 10-20 ml of each sample was harvested every 2 days and centrifuged for 15 min at 10,000 rpm for the separation of supernatant. Then the supernatant was filtered through bacterial filter (Millipore filter 0.45µm) to get cell free samples. Antimicrobial activity of each cell free supernatant was determined using well diffusion assay (Schillinger and Lucke, 1989), 50 to 200µl of sample was loaded and tested. Antimicrobial activities were tested against all the test microorganisms.

Extraction of antimicrobial compounds

The antimicrobial compound was recovered mainly from the cultured broth was extracted with ethyl acetate and concentrated at pH 8.0 and after the evaporation of solvent the remaining residue was collected as antimicrobial compound.

Purification of antimicrobial compounds

The obtained antimicrobial compound was purified by silica gel column Chromatography. Two grams of crude power was dissolved in 10 ml ethyl acetate. The solution was passed through a silica gel column in benzene. The TLC plate placed vertically in a trough (solvent chamber) containing suitable solvents (n-butanol-ethyl acetate-water 9:9:1). When the solvents moved up to 80 % of TLC plate, the plate was taken out, dried and sprayed with Ninhydrin.

Characteristics of antimicrobial compounds

The solubility of the antimicrobial compounds of the actinomycetes was tested using various solvents such as ethyl acetate, methanol, chloroform and distilled water. The ultra violet spectral measurement of the ethyl extract of purified compound from fermentation broth was made at 200-400 nm by Hitachi (UV 1601) instrument, using the methanol as solvent (Swaadoun *et al*., 1999).

Antimicrobial assay of partially purified compound

Antimicrobial property of the pure compound was determined by well diffusion assay methods using the bacterial pathogens such as *B. subtilis, S. aureus, E. coli and Kl.pneumoniae.* The compounds was dissolved in solvent namely, ethyl acetate. The 5 mm diameter well was made using a cork borer. The final mixture was poured in to the well separately and incubated at 37°C for 24 hours.

RESULTS AND DISCUSSION

Actinomycetes population diversity of sea shore soil sample

A total of four actinomycetes isolates were observed as morphologically distinct colonies on Starch casein agar. All the actinomycetes isolates are belonging to three genera in which most of them belonged to the genus *Streptomyces* sp.

Characterization and identification of actinomycetes

Among the four isolated actinomycete only one actinomycete possess a broad spectrum antimicrobial activity. The different parameters namely, morphological, biochemical characters were used for the identification of actinomycetes isolate.

Morphological characterization

The normal microscopic view of actinomycete showed tight spirals of smooth spore surface, aerial and substrate mycelium and diffusible extracellular pigments (table-1).

S.No	Properties	Actinomycetes
1	Sporophore morphology	Spirally twisted
2	Spore surface	Smooth
3	Colour of aerial mycelium	Dull white
4	Colour of substrate mycelium	Dull yellowish brown
5	Spore mass	White
6	Gram staining	+ve
7	Acid fast	Non acid fast

Table 1-Characterization and identification of *Streptomyces species*

Biochemical characterization

S. No	Tests	Result
1	Indole test	Negative
2	Methyl red	Negative
3	Voges proskauer test	Negative
4	Citrate utilization	Positive
5	Catalase test	Negative
6	Oxidase test	Positive
7	Starch hydrolysis	Positive
8	Casein hydrolysis	Positive

Table 2- Biochemical characteristics of *Streptomyces species*

Primary screening

Out of all the isolated actinomycetes, an efficient actinomycete isolate showed the good antibacterial activity by cross streak method was screened for further characterization

Secondary screening

Two media of Nutrient broth (NB) and Starch casein broth (SCB) were tested for the production of antimicrobial substances producing activities. Results indicated that Starch casein broth (SCB) produced higher efficiency of novel metabolites. Antagonistic activity was measured at different incubation periods. A maximum activity was measured at the period of 7th day. The rate of antimicrobial activities was correlated with zone of inhibition and was highest in the late log phase. The pH of the broth was within the range of 6.9 ± 7.2 through out the fermentation.

Antimicrobial efficacy

The ethyl acetate extract of fermented broth of *Streptomyces* species isolate was tested against four pathogenic bacteria's. The maximum inhibition was observed against *Staphylococcus aureus* (35±0.56 mm) followed by *Bacillus subtilis* (25±0.34mm), *Escherichia coli* (25±0.34 mm) and *Klebsiella pneumoniae* (9±0.24 mm) (table-3).

S.No	Target organisms	Zone of inhibition (mm)
1	*Bacillus subtilis*	25±0.34
2	*Staphylococcus aureus*	35±0.56
3	*Escherichia coli*	20±0.38
4	*Klebsiella pneumoniae*	9±0.24

Table 3- Zone of inhibition (mm) of various human pathogenic bacterias

Characterization of antimicrobial compounds

The antimicrobial compound prepared using ethyl acetate were purified and separated by column and thin layer chromatography. Single separated band was observed in the sample. The Rf value of *Streptomyces* species produced compound was 0.40 in thin layer chromatographic- separation. *Streptomyces* species compound revealed that the absorption maximum was 200 to 295 nm in ethyl acetate solvent. The UV spectrum of *Streptomyces* species is shown in (Fig.1).

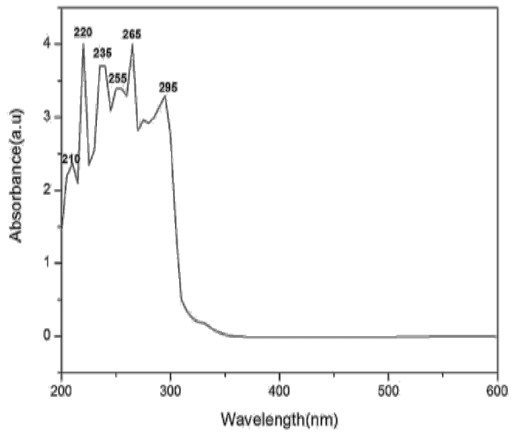
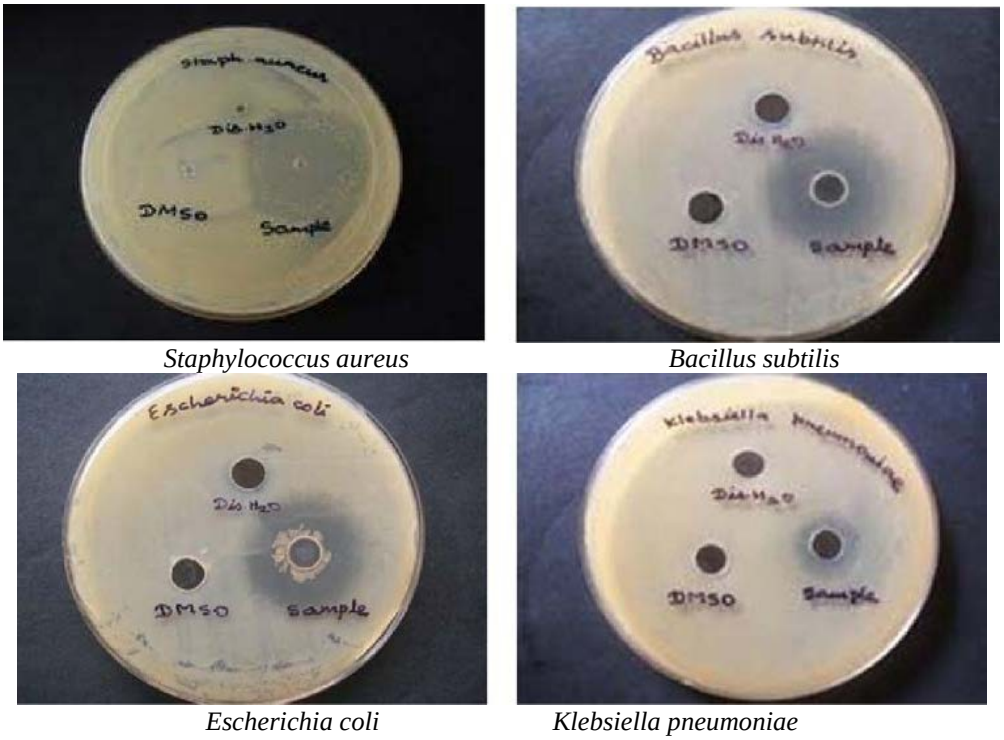


Fig.1- UV-Spectrum of partially purified compound from *Streptomyces* species

Antimicrobial activity of purified compound of *Streptomyces* species

The purified antimicrobial compounds of *Streptomyces* species showed maximum inhibitory effect against *Staphylococcus aureus* (38±0.44 mm) followed by *Bacillus subtilis* (29±0.36 mm), *Escherichia coli* (25±0.28 mm) and *Klebsiella pneumoniae* (11±0.24 mm)(fig.2)



Staphylococcus aureus *Bacillus subtilis*

Escherichia coli *Klebsiella pneumoniae*

Fig.2- Antimicrobial activities against selected pathogenic microorganisms

CONCLUSION

The present study showed that the antibacterial efficacy of *Streptomyces* species was maximum against *Staphylococcus aureus* (38±0.44 mm) followed by *Bacillus subtilis* (29±0.36 mm), *Escherichia coli* (25±0.28 mm) and *Klebsiella pneumoniae* (11±0.24 mm). The antimicrobial efficacy of compounds from *Streptomyces* species has been studied against clinical pathogens (Wu and Chen, 1995; Patil *et al*., 2001). Further, work in improving and isolation strategies in the discovery of marine microorganisms, purification and identification of exact compounds are of utmost importance for ensuring success in this area. Marine microorganisms represent an underexploited source of the discovery of novel secondary metabolites and their potentials should not be overlooked.

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