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SCREENING AND ANTIMICROBIAL STUDIES ON BIOACTIVE COMPOUNDS PRODUCED BY MARINE ACTINOMYCETES

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ABSTRACT: The study concerns with actinomycetes isolated from different marine soil with special reference to antimicrobial substances. Total of five morphologically different actinomycetes strains were isolated from marine soil collected from three different locations, India. The screening method was performed for the five strains in which strain ABTRI 2 showed inhibitory activity against the set of test pathogens. Bioactive substance from strain ABTRI 2 was produced by adopting fermentation process and extracted using ethyl acetate. In well diffusion method, ethyl acetate extract showed potent antibacterial activity (11-22 millimeter zone of inhibition). Active fraction present in the ethyl acetate extract was determined by thin layer chromatography. Findings of this work revealed that the marine ecosystems investigated in this study will be potential place for promising applications in the environment.

Keywords: Fermentation process, antibacterial activity, crude compound, TLC

INTRODUCTION

Microorganisms are attractive sources of bioactive compounds with pharmaceutical and agricultural importance. Marine microbes encompass all microscopic organisms generally found in salt water. Marine microbes play many important roles in the earth and they are the major primary producers in the ocean, and provide us with a source of medicines and natural products. Natural products remain to be the most propitious source of antibiotics.

The biodiversity of marine environment is an important resource for isolation of potent microorganisms to produce biologically active secondary metabolites (antibiotics), which having both structural / chemical features. Substances which are biologically active against diseases causing pathogens are naturally present in microorganisms, plants and animals. Marine Actinomycetes are filamentous, gram positive prokaryotes; it has the intermediate characteristics of bacteria and fungi. These are the most economically and biotechnologically valuable prokaryotes.

Actinomycetes are used for the production of bioactive secondary metabolites (Chaudhary *et al.,* 2013), specificallyantibiotics,anti-tumoragents, imunosuppressive agents, antiparasitic agents, enzymes, vitamins, nutritional materials and these metabolites are used as cosmetics, herbicides pesticides and other valuable products (Attimarad *et al*., 2012; Valli *et al*., 2012).

Several novel bioactive compounds were discovered from aquatic actinomycetes, for example rifamycin from *Micromonospora* species salinosporamide-A, anticancer metabolite from *Salinispora* species, marinomycins from *Marinophilus* species, abyssomicin-C from *Verrucosispora* species, and marinopyrroles from *Streptomyces* species, anticancer metabolite from *Salinispora* species. Abyssomicin C possesses potent activity against Gram positive bacteria, including clinical isolates of multiple drug resistant pathogens.

MATERIALS AND METHODS

Sample collection and Isolation

The soil samples were been collected from kovalam, thiruvanmiyur and mamallapuram seashores and the soil samples were pre-treated

with 0.1g Calcium carbonate($CaCo_3$). The marine actinomycetes were isolated by serial dilution technique followed by spread plate method. The selected strains was maintained on Starch Casein Agar (SCA) medium and also maintained in slant cultures. They were named as ABTRI 1, ABTRI 2, ABTRI 3, ABTRI 4, ABTRI 5.

Preliminary screening against Human Pathogens

Starch casein Agar and Nutrient agar was prepared in equal volume, sterilized and poured into sterile petriplates by mixing both the media and allowed to solidify. The actinomycete strains (ABTRI 1-5) were inoculated (5cm measurement) on each plate by single streak method. The plates were incubated for 4-5 days. 24 hours cultures *Staphylococcus aureus, Proteus vulgaris, Micrococcus luteus* and *Klebsiella pneumoniae* were streaked (3cm measurement) by perpendicular streaking method, incubated overnight and observed for inhibition. Similarly, antifungal activity was screened against *Aspergillus niger* (disc) using SCA and PDA plates where SCA and PDA were used in equal ratio.

Extraction of Secondary metabolites by Fermentation process

The secondary metabolite production was been processed for extraction of crude compounds by ethyl-acetate extraction method. The selected strain based on screening results was been inoculated in ISP-2 broth and the broth was kept in an orbital shaker for incubation for 10days in order to reach maximum production. After incubation, the culture broth was filtered and centrifuged at 10,000 rpm for 15 minutes and the supernatant was collected and mixed with an equal volume of ethyl acetate. The extracted crude compounds were dried at 40°C. The crude compound was been processed for chromatographical technique to confirm the presence of bioactive metabolites.

Thin layer chromatography

TLC is a chromatography technique used to separate non-volatile mixtures (Harry *et al.,* 1989). TLC is a solid-liquid system of chromatography where the stationary phase was the silica gel and the mobile phase was Methanol : Chloroform : Toluene solvents.

Calculation of Rf value:

Rf value = Distance travelled by solute / Distance travelled by solvent

Qualitative bioassay of the compound - Screening for antibacterial activity by Well Diffusion method

Nutrient agar was prepared and 24hours culture *Staphylococcus aureus, Proteus vulgaris, Micrococcus luteus* and *Klebsiella pneumoniae* were swabbed on it. Then, five lawns were made using sterile cork borer. Various concentrations (250µg-1000µg) of test sample (10mg/ml DMSO) was been loaded in wells. Tetracycline was been used as control. The plates were then incubated at 37ºC for 24h. After incubation the inhibition diameter was measured.

RESULTS AND DISCUSSION

Subculture process

The isolated colonies were subcultured on Starch Casein agar plates and were named as ABTRI 1, ABTRI 2, ABTRI 3, ABTRI 4 and ABTRI 5.

Preliminary screening against Human Pathogens

The ABTRI 1-5 were tested for their antibacterial activity by perpendicular streaking. From the five strains, ABTRI 2 was selected as an effective strain based on inhibition. The zone of inhibition for ABTRI 2 against *Proteus vulgaris* was recorded as 7.5cm. The ABTRI 2 was effective and so it was selected to check the antifungal property. The ABTRI 2 doesn't exhibit antifungal activity against *Aspergillus niger.*

In the current study marine actinomycete strains were isolated from soil samples collected from Kovalam, Mammalapuram and Thiruvanmiyur beaches. A similar study on isolation of antibacterial actinomycetes was carried out by Devi *et al*., (2006). It has been reported that 10 strains were isolated from marine water collected from Dhanushkodi, among which, 3 strains were found to be *Actinopolyspora*, *Nocardia* and *Streptomyces* sp. The *Streptomyces* species showed best level of antibacterial activity against *S. aureus*, *P. aeruginosa*, *K. pneumonia*, *S. typhi* and *A. niger*. Whereas, the other 2 species *Actinopolyspora* and *Nocardia,* showed antifungal activity only. Totally 164 strains were isolated from 39 sediment samples collected from the Bay of Bengal coast of Puducherry and Marakkanam and the isolate VITSVK9 showed antibacterial activity against *Bacillus subtilis* (18 mm) and antifungal activity against *Aspergillus niger* (17 mm) (Thenmozhi and Kannabiran, 2012). The

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results of the current study revealed that the isolated strains possessed significant antibacterial activity and no significant inhibition of fungal strains.

Extraction of Secondary metabolites by Fermentation

After 24 hours of the ethyl acetate extraction method, the solvent layer was collected and condensed to obtain the crude extract. This resulted in yellow coloured, thick viscous extract.

Thin layer chromatography

Partial purification of secondary metabolites was done by TLC method. Active compounds were identified based on the optimized solvent system(Methanol:Chloroform:Toluene).

T	Zone of inhibition (mm)				
	C	250 µg/ml	500 µg/ml	750 µg/ml	1000 µg/ml
A	24mm	11mm	14mm	15mm	18mm
B	24mm	11mm	20mm	21mm	22mm
C	17mm	11mm	12mm	15mm	17mm
D	18mm	12mm	13mm	15mm	15mm

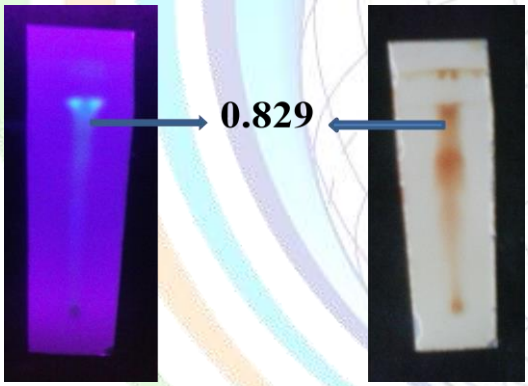

Fig. 1: TLC Profile (Under UV and Under Iodine) for ABTRI 2

The TLC profile of the crude extract of ABTRI 2 showed distinct band with R_f value 0.829, under UV illumination. These compounds might be responsible for antimicrobial activity of crude extract.

The bioactive compounds isolated from the strain ABTRI 2, when subjected to TLC, showed the presence of distinct compounds with R_f values 0.829. A similar study on the extracts obtained from *Streptomyces* isolated from sediment soil showed nine bioactive regions with Rf values from 0.21 to 0.96 (Ilic *et al.,*2005). Similar result was obtained from the cultural extracts of marine sponges associated *Streptomyces* was reported (Dharmaraj and Sumantha, 2009). IR spectra of crude extract showed some different vibrational peaks of these functional groups in the extract depicts that the diverse activity they exhibited against test organisms during the susceptibility screening. The distribution of the antibiotic inhibition phenotype of *Streptomycetes* with great antibacterial and antifungal activity which gave a similar spectra profile has also been reported (Illic *et al*., 2007). Nevertheless, further investigation is needed in order to purify and determine the structure of the active components in the extracts.

Qualitative bioassay of the compound - Screening for antibacterial activity by Well Diffusion method

The extracted bioactive compound was subjected to well diffusion assay to study its antibacterial property against *S.aureus*, *K.pneumoniae*, *P. vulgaris* and *M.luteus*. The compound showed maximum inhibition against *K.pneumoniae* with ZOI of 22mm at a concentration of 1000 µg/ml.

Table 1: Antibacterial activity for the bioactive compounds against human bacterial pathogens

T-Test,

A-*Staphylococcus aureus*

B-*Klebsiella pneumoniae,*

C-*Proteus vulgaris,*

D-*Micrococcus luteus*

Actinomycetes, "the reservoir of secondary metabolites and enzymes", dominant and significant microbes inhabiting soil environment comprises about 50% of the uncultivable soil microbes. The immense source of novel metabolites and their therapeutic applications, specifically as drug lead molecules serves as a natural blue print for developing new drugs. Fungal drug resistance is a growing problem worldwide and search for new and effective antifungals to overcome drug resistance is of current importance. Actinomycetes isolate producing polyene type of metabolite has been reported to be effective against pathogenic bacteria and fungi.

A polyketide antibiotic extracted from *Streptomyces* sp. AP-123 have been shown to be very effective against *C. albicans* and filamentous fungi *A. niger*. New triazole antifungal drugs and combination drugs are underway to overcome invasive fungal infections and emergence of resistance. Penicillin, Tetracycline, cephalosporins are important soil derived antibiotics. Vancomycin (isolated in 1956) from actinomycete species found in Indian and Indonesian soils, is very powerful and currently serves as the last line of defense to treat bacterial infections. Actinomycetes (C11 and C12) isolated from marine environment has been shown to be effective against group of bacterial pathogens. Marine actinobacteria isolated from salt pan environment, SRB25 has been reported to be effective against multidrug resistant *Staphylococcus aureus* (MDRSA). However, the results obtained from the current study revealed that the isolated strains produced bioactive compounds possessed effective antibacterial activity against human pathogens but it did not possess significant antifungal activity against *A.niger* (Subashini and Kannabiran, 2013).

CONCLUSION

From the present study, it is evident that ABTRI 2 could exhibit antibacterial activity and no inhibition against fungal pathogen. The crude compound (from ABTRI 2) extracted from ethyl acetate was effective and had potent antibacterial activity against various bacterial pathogens such as *Staphylococcus aureus, Klebsiella pneumoniae, Proteus vulgaris* and *Micrococcus luteus*. It can be concluded that the isolated strain has been found to possess appreciable antimicrobial activity. The presence of bioactive compounds was been confirmed by thin layer chromatography. Hence, the bioactive compounds play promising mechanism in the environment with various applications.

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