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CHARACTERIZATION OF HALOPHILIC ANTAGONISTIC ACTINOMYCETES ISOLATED FROM SOLAR SALTPAN OF TAMILNADU, INDIA.

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ABSTRACT

The actinomycetes are existing in various habitats in nature. The terrestrial forms have been extensively used for the production of secondary metabolites and used to treat infectious diseases. However the extreme counterparts have reminded relatively unknown and unexploited. The soil from the saltpan regions of Cuddalore, Parangipettai and Portonovo Tamilnadu, India were screened for the isolation of antagonistic actinomycetes. In the present study, morphologically diverge four actinomycete isolates were obtained from different sampling station were identified as Streptomyces species. All the four actinomycetes were screened for preliminary antibacterial activity. Out of four strains screened, one strain Streptomyces sp SBU1 showed most promising antagonistic activity against Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus. Secondary screening of the actinomycetes ethyl acetate extract also exhibited optimistic antibacterial activity against P.aeruginosa (22 mm) followed by S.aureus (19 mm) and E.coli (18 mm). Partially purified column chromatography fractions (fraction 1 and 2) showed significant antimicrobial activity against P.aeruginosa (16 mm). A single separate band of the antimicrobial compound was observed by TLC. The Rf value of fraction 1 and fraction 2 was 0.83 and 0.75 respectively. The present investigation indicates that the solar saltpan soil harbors diverse species of actinomycetes possessing potent antibacterial activity.

KEYWORDS: solar saltpan, antagonistic actinomycetes, clinical pathogens, Streptomyces sp.

INTRODUCTION

Actinomycetes are the group of gram positive, filamentous bacteria from diverse ecological niches is known to produce chemically diverse compounds with wide range of biological activities (Bredholt et al., 2008). Actinomycetes population has been identified as one of the major group of soil population which may vary with the soil type (Agarwal, 2002). They are originally considered as an intermediate group between bacteria and fungi. They have high Guanine plus Cytosine (70 – 74%) content in their DNA (Korn-Wendisch and Kutzner, 1992). 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). They are free living, saprophytic bacteria, and a major source for production of antibiotics. Streptomyces are prolific and can produce a great many antibiotics and was estimated about 80% of medically important antibiotic producers (Mellouli et al., 2003). Streptomyces is the largest antibiotic genus, producing both antibacterial and antifungal and also a wide range of other compounds such as immunosuppressant (Okami et al., 1978). The terrestrial forms have been extensively used for the production of secondary metabolites and used to treat infectious diseases. Though there are few reports found in literature specifically with respect to bioactive compounds from salt pan isolates (Tonima K. Kamat and Savita Kerkar 2011). However the saltpan and the marine Streptomyces counterparts reminds largely unexploited for useful metabolites. As the human pathogens developed resistance to antibiotic drugs, there is an urgent need for the safe, nontoxic and cost effective antibiotics. In the present investigation soil samples from various saltpans of Cuddalore, Parangipattai and Portonovo Tamil Nadu, India were examined for the presence of antagonistic actinomycetes and the most promising isolates thus obtained were tested for the production of antibacterial metabolites.

MATERIALS AND METHODS

Sample collection:

Sediment soil samples were collected from saltpan in three different stations namely Cuddalore (Lat 11° 45' N and Long 79° 47' E), Parangipattai (Lat 11° 28 N Long 79° 44’ E) and Portonovo (Lat 11° 28 N Long 79° 43’ E) in and around Tamil Nadu. The samples were collected 2 inches from the soil surface to avoid cross contamination using sterile spatula and transferred into sterile polypropylene bags and brought to the laboratory for microbiological analysis. In order to eliminate other non spore forming bacteria and fungi, the sediment samples were pre treated with calcium carbonate (10:1 w/w) in room temperature for four days. Then the samples were further dried at room temperature for about three weeks were subjected for the enumeration of actinomycetes (Gurung et al., 2009).

Microorganism and culture media:

The actinomycetes, Streptomyces sp. was isolated from sediment sample by spread plate method using Actinomycetes isolation agar (AA) Hi-media, Mumbai, India in aged seawater and distilled water (1:1) ratio (Okazaki et al., 1983). The inoculated agar plates were incubated at 28^{o}C for 7 days. A typical powdery colony was picked and sub-cultured on yeast extract malt agar (ISP2 medium; International Streptomyces project) containing Glucose 5g; yeast extract 4g; malt extract 10g; agar 18g; aged sea water 500ml and distilled water 500ml; pH 7.0±0.2 . The isolate was gram positive, having a

long filamentous structure and are identified as Streptomyces species based on the morphological, physiological and biochemical characteristics on ISP2 media [Shirling and D. Gottileb, 1966].

Test organisms

Clinical isolates of gram positive organism Staphylococcus aureus and gram negative organism such as Escherichia coli and Pseudomonas aeruginosa were obtained from Sharp Clinical Lab, Perambur, Chennai for the present study.

Primary screening of antagonistic actinomycetes:

The antagonistic activity was tested by Cross streak assay method (Ellaiah et al., 1996). Single streak (6 mm diameter) of isolated actinomycete strains were streaked on the surface of Modified Nutrient Agar (MNA) containing Glucose 5g; Peptone 5g; Beef extract3g; Sodium chloride 5g; Agar 18g; aged sea water 500ml and distilled water 500ml. Final pH of the medium was adjusted to 7.0±0.2 and autoclaved at 15lbs for 15 minutes. The plates were incubated at 28±2^{o}C for 5-7 days. After attaining a ribbon like growth, the over night culture of selected pathogens were streaked at perpendicular to the original streak of actinomycetes and incubated at 37 ^{o}C for 24 hours. Triplicates were maintained for each isolates. Control plate was also maintained without inoculating actinomycetes to evaluate the normal growth of pathogens. Bioactive metabolites production was determined in terms of their antimicrobial spectrum. The antagonistic actinomycete which showed outstanding broad spectrum activity was selected for further secondary screening.

Secondary screening of actinomycete metabolites:

The most promising strain was subjected for the mass cultivation of antimicrobial metabolites extraction. A loop full of selected actinomycetes inloculum was inoculated into 250 ml Erlenmeyer flask containing 100ml of yeast extract malt broth (ISP2) and incubated in an orbit shaker incubator (NEOLAB) with 200 rpm at 28±2^{o}C for 48 hours. Ten milliliter of the seed culture was transferred to the 1000 ml of Soyabean Meal Broth (SMB) and incubated at 28±2^{o}C in a shaking incubator (200 rpm) for 5-7 days. After incubation the cell free filtrate was mixed with equal volume of ethyl acetate (1:1 V/V) in a separating funnel for the extraction of bioactive compound. These mixtures were shaken vigorously for 10 minutes and leave it for 30 minutes in an undisturbed condition for complete extraction. The lower aqueous phase was discarded and upper organic phase was concentrated in a vacuum evaporator at 40^{o}C for 24 hours to obtained crude extract used to determine the antimicrobial activity. The crude extract was subjected to perform antimicrobial sensitivity assay method followed by Bauer et al (1966).Control plates were maintained by the discs amended with ethyl acetate and all the plates were incubated at 37 ^{o}C for 24 hours. Triplicates were maintained for each clinical pathogen. The zone of inhibition appearing around the discs were measured and recorded as millimeter in diameter.

Purification of crude actinomycete metabolites:

The residue was dissolved in an appropriate volume of ethanol and used for further purification. The crude extract was purified by silica gel column chromatography (SGCC) using silica gel (60 – 120 mesh) as a stationary phase and ethanol: water (1:1 ratio V/V) as a mobile phase. The column (35 X10

mm) was cleaned and packed with silica gel with sterile distilled water. The metabolite extracts were partially purified by elution with (10% stepwise) 0–100% by volume of ethanol in water through a silica gel column. The fractions were collected every 20 minutes interval. Each fraction was analyzed by thin layer chromatography (TLC) using pre-coated silica gel plates of 0.25 mm thickness (Merck, India) in order to identify the fraction that contained metabolites. Ethanol, water and chloroform were used in a volume ratio of 90:25:4 to develop the chromatogram. Each fraction was subjected to perform antimicrobial sensitivity assay against selected clinical pathogens. The eluted spots in the plate were visualized in the iodine vapour chamber and the Rf value of partially purified metabolite was calculated by the following formula.

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

RESULTS AND DISCUSSION

Microbes from solar saltpans are yet to be fully explored as potential producers of antimicrobial agents. The actinomycetes isolated from solar saltpan are subjected to genus level identification. The colony morphology and cell wall type of our isolates under investigations belonged to the genus Streptomyces. All the isolates were identified as Streptomyces sp.-SBU1; Streptomyces sp.-SBU2; Streptomyces sp.-SBU3 and Streptomyces sp.-SBU4 based on the micro-morphological, physiological and biochemical characteristics (Table 1). The isolates posses profusely branched long filamentous structure with aerial mycelium and substrate mycelium. Aerial mycelium contains globes, smooth terminal spores. All the strains isolated from saltpan could grow up to 45^{o}C. LL- diaminopimelic (LL-DAP) was present in the cell wall along with glycine. That indicates the cell wall chemo-type-1, which is the characteristic of Streptomyces species. Melanin pigment and reverse side colour were noticed for the strain SBU1, SBU3 and SBU4. Deepika and Kannabiran (2009) have been reported that actinomycetes isolated form Ennor salt pan region belongs to Streptomyces species. Dhanasekaran et al., (2005) have been indicated that the species of Streptomyces and Saccharomonospora are dominant in the saltpan of Cuddalore, Tamil Nadu. The actinomycetes isolated from saltpan were subjected to preliminary screening against selected clinical pathogens to choose the potential antagonistic actinomycetes. It revealed that, out of four actinomycetes strains were tested, Streptomyces sp.-SBU1 exhibited promising antagonistic activity against all the tested pathogens (Table 2). Under stress condition the organisms are bound to elaborate several different chemical entities for their survival (Chiaki et al., 2007) and those secondary metabolites are used for several potential applications. However, few reports are available on the antimicrobial potential of actinomycetes isolated from Indian saltpan. Suthindhiran K and Kannabiran K (2009) have reported that Saccharopolyspora salina VITSDK4, isolated from a saltpan marine soil sample collected at the Marakkanam coast of the Bay of Bengal, India, was profoundly antagonistic with fungal and gram positive pathogens. Dhanasekaran et al., (2008-2009) have reported three actinomycetes, from saltpan regions of Cuddalore and Parangipettai, Tamil Nadu, India, showing promising antibacterial activity against tested organisms. Dhanasekaran et al., (2005) have also reported that out of nine isolates six Streptomyces sp (66.7%) isolated from saltpan soil exhibited broad spectrum antibacterial activity. In the present study 75% of isolate showed antagonistic activity against at least one of the selected pathogens. The most promising potential strain was adopted for mass scale production of secondary metabolites. The antimicrobial susceptibility of crude ethyl acetate extract was adopted by disc diffusion method against clinical pathogens and is represented in fig.1. The maximum zone of inhibition was noticed against P.aeruginosa (22 mm) followed by S.aureus (19 mm) and E.coli (18 mm).

The crude ethyl acetate extract of actinomycete metabolites was partially purified by column chromatography. In the present research column chromatography fractions were separately collected and performed thin layer chromatography to check the purity and to calculate the retention factors (Rf) value of the metabolite. A single separate band of the antimicrobial compound was observed by TLC. The Rf value of fraction 1 and fraction 2 was 0.83 and 0.75 respectively. The fluorescence colour of the spot was brownish yellow. All the fractions were subjected to antimicrobial susceptibility against selected clinical pathogens. Of the eight fractions tested, fraction 1 exhibited antimicrobial activity against all the pathogens. Fraction 2 showed antimicrobial activity against P.aeruginosa and S.aureus. There is no activity against E.coli. Amusingly both the fractions showed maximum activity (16 mm) against P.aeruginosa (Table 3). Fraction 3 to Fraction 8 observed no activity against tested clinical pathogens. Gurung et al., (2009) reported that, the Rf value of crude extract showed two spots. The retention factors (Rf) of moved spot were 0.88 for the isolate K.6.3, 0.86 for the isolate K.14.2 and 0.95 for the isolate K.58.5. Vijayakumar et al., (2011) observed that, the Rf value of the compound produced by sp. Streptomyces VPTSA18 was 0.48 by TLC chromatogram. The colour of the separated antimicrobial compound was reddish brown. Earlier research supported that antimicrobial metabolites extracted from actinomycetes isolated from solar saltpan showing comparable Rf value of the present investigation. Many researchers have tested the antimicrobial efficacy of the compounds obtained from actinomycetes isolated from extreme habitat against a variety of clinical pathogens. It has been reported that the antimicrobial activity of the compounds from different strains of actinomycetes vary depending on the strains from which the compound obtained. This research opens a new avenue for exploration of other solar saltpans for the discovery of potential antagonistic halotolerant and halophilic actinomycetes as pharmaceutically important microorganisms from extreme habitat.

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Characteristics	Name of the actinomycetes			
	SBU1	SBU2	SBU3	SBU4
True mycelium	Present	Present	Present	Present
Aerial mycelium colour	Red	White	Grey	Yellow
Reverse side colour	Red	-	Brown	-
Melanin pigment	+	-	+	+
Facultative anaerobe	+	+	+	+
Spores in aerial mycelium	+	+	+	+
Spores in substrate mycelium	-	-	-	-
Sporangium on aerial mycelium	-	-	-	-
Spore chain morphology	Recti-Flexible	Recti-Flexibles	Recti-Flexible	Recti-Flexibles
No. of spores on aerial mycelium	20 – 50	13-20	20-50	15-17
Spores shape	Globes	Globes	Globes	Globes
Spore surface	Smooth	Smooth	Smooth	Smooth
Cell wall type	1	1	1	1
DAP isomer	LL-DAP	LL-DAP	LL-DAP	LL-DAP
Growth at 45^0C	+	+	+	+
Alkalophilic	+	+	+	+
Halophilic	+	+	+	+

DAP – Diaminopimelic acid ; + : denotes Positive; - : denotes Negative; Streptomyces sp.-SBU1; Streptomyces sp.-SBU2; Streptomyces sp.-SBU3; Streptomyces sp.-SBU4

Table 1: Genus level identification of actinomycetes isolated from solar saltpan.

Isolates	Selected clinical pathogens.		
	E.coli.	P.aeruginosa	S.aureus
Streptomyces sp.-SBU1	+++	+++	+++
Streptomyces sp.-SBU2	++	-	-
Streptomyces sp.-SBU3	+	+	+
Streptomyces sp.-SBU4	-	+	-

+++: excellent activity; ++: good activity; +: moderate activity; - : no activity

Table2: Antagonistic activity of isolated actinomycetes against selected pathogens.

Antimicrobial Metabolites	Zone of inhibition in mm diameter		
	E.coli.	P.aeruginosa	S.aureus
Fraction 1	12	16	12
Fraction 2	-	16	14
Fraction 3	-	-	-
Fraction 4	-	-	-
Fraction 5	-	-	-
Fraction 6	-	-	-
Fraction 7	-	-	-
Fraction 8	-	-	-

-: no activity

Table3: Antimicrobial susceptibility of partially purified actinomycete metabolites against selected pathogens.

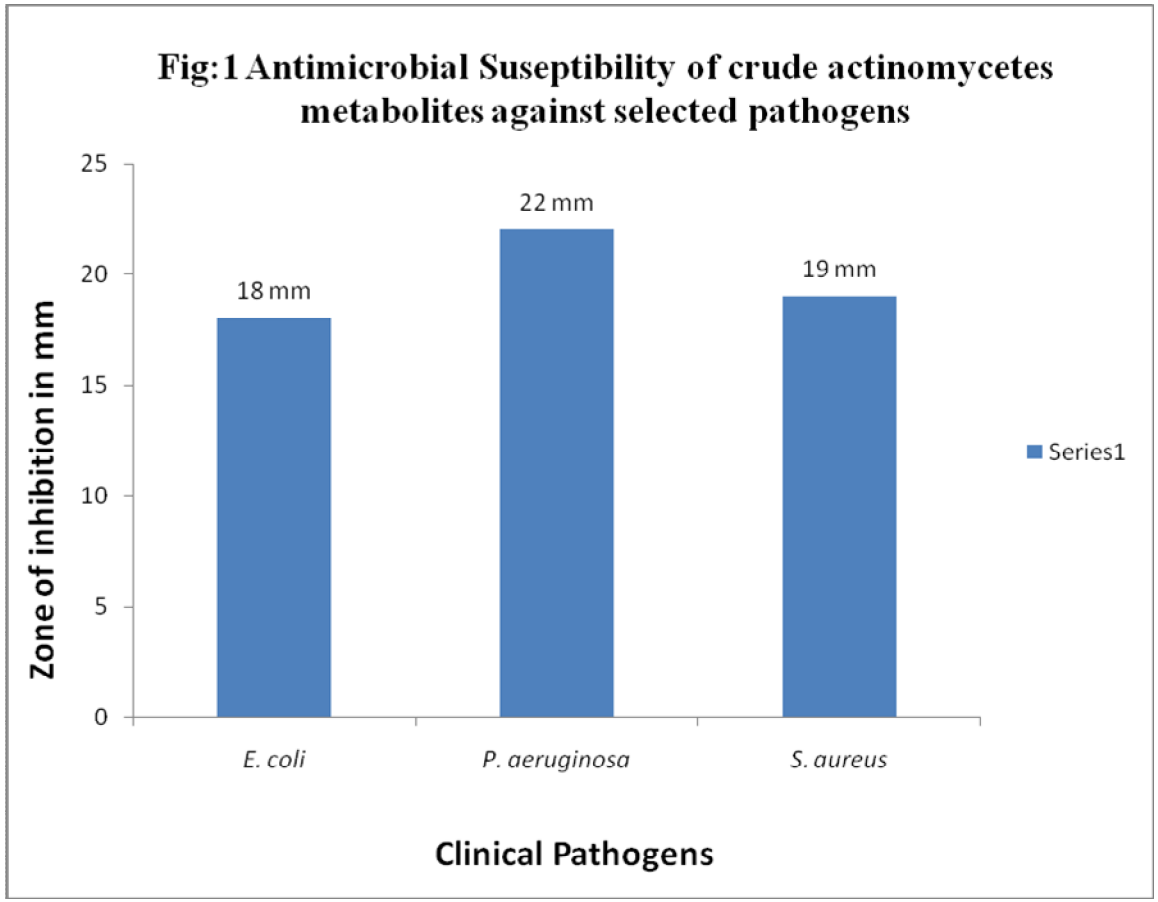
Fig:1 Antimicrobial Suseptibility of crude actinomycetes metabolites against selected pathogens
25
20
15
10
5
0
Zone of inhibition in mm
18 mm
22 mm
19 mm
E. coli
P. aeruginosa
S. aureus
Clinical Pathogens
Series1