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RES3EARCH ARTICLE

ANTIMICROBIAL ACTIVITY OF CRUDE ETHYL ACETATE EXTRACT OF MARINE *Actinomycetes* ISOLATED FROM MARINE SEDIMENTS

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ABSTRACT

Actinomycetes are wide spread in nature and known for production of bioactive metabolites. Marine source world-wide are considered to be rich for bio-resource and efforts has been taken to identify new bacterial agents from marine environment for human welfare. In this study, we reported isolation of 20 *Actinomycetes* strains from marine sediments and tested with standard biochemical test and utilization of carbon sugar, to confirm the *Actinomycetes* character. Among the isolates 17 and 3 were recorded to be gram positive and gram negative respectively. Carbon utilization test revealed that all the isolates utilized dextrose and none of the isolates utilized ducitol. Antagonistic activity of 20 strains revealed that six strains (PS2, PS4, PS9, PS11, PS13 and PS14) are positive and crude ethyl acetate extracts of strains PS4, PS11, and PS14 were showed good antimicrobial activity. The isolate PS14 dominant over other isolates and revealed promising antimicrobial activity.

INTRODUCTION

Actinomycetes are gram positive bacteria that are wide spread in nature and play a pivotal role in the production of bioactive metabolites (Sanglier *et al*., 1993). *Actinomycetes* especially *Strepotmyces* are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites, such as antibiotics, antitumor agents (Rofiq sunaryanto *et al*., 2010). Among the potential sources of natural products bacteria have prove to be a particularly prolific resource whilst a surprisingly small groups of taxa accounting for the vast majority of compounds discovered. For example, of the 53 known bacterial phyla only five are reported to produce anti-infective agents (Keller and Zengler 2004). Among these five, the class action bacteria and more specifically the bacteria belong to the order *Actinomycetales* (commonly called *Actinomycetes*) account for approximately 7000 of the compounds reported in the dictionary of natural products. Looking individually at the more than 140 currently described *Actinomycetes* genera, it become clear that with order, it is few well known soil genera that account for the vast majority of microbial natural products discovered. In fact the genus *Streptomyces* alone accounts for a remarkable 80% of the *Actinomycetes* natural products reported to date a biosynthetic capacity that remain without rival in the microbial world. Baam *et al*. (1966), isolated two antagonistic *Streptomyces sp*. from Bombay water. Okazaki *et al*. (1975) reported a new antibiotic compound SS-228Y from China species isolated from the Sagami Bay, having antibacterial and anticancer activities. While studying the Sagami Bay marine sediment, Okami *et al*. (1976) isolated Streptomyces griseus, which produced a new antibiotic Aplasmomycin, which inhibits gram positive bacteria including mycobacteria in vito and plasmodia *in vivo.* Okami *et al*. (1979) isolated a new species Streptomyces tenjamariensis from shallow sea mud around Sagami Bay, which produced antibiotics isamycin A and B with strong antibiotic activity against gram positive and gram negative bacteria. A logical extension for the search of *Actinomycetes* natural products is the study of marine derived strains. Although these strains appear to be a useful source of new molecules with more than 100 compounds described to date, it was only demonstrated that some were in fact indigenous to the marine environment and not merely transient contaminants from shore (Blunt *et al*., 2004). Give the large numbers of *Actinomyctes* are undoubtedly washed from shore onto the sea distinguishing between those that have evolved in response to specific marine environmental challenges vs. those that are present as dormant spores must clearly be a priority if we to understand how life in the marine environment affects secondary metabolism. Although few natural products have assessed the taxonomic novelty of marine derived strains those that have yielded. In the present study, we are isolated *Actinomycetes* strains from marine sediment, these strains were

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tested for biochemical characterization, utilization of carbon sugar and antimicrobial activity of crude ethyl acetate extracts.

MATERIALS AND METHODS

Reagents

Sediment samples were collected from shrimp farms near Parangipettai, Tamil Nadu, India. Solvents like Hexanes, Ethyl acetate, Acetone, Chloroform and Methanol were obtained from Merck, Germany. Muller Hinton Agar (MHA), Nutrient Agar, Nutrient Broth, Tryptone broth, SIM agar, Starch agar, Kovac's reagent, Crystal violet, Oxidase disc, Gelatin agar, Sulphanilic acid and α-naphthylamine reagent were purchased from Himedia Laboratories, Mumbai, India.

Isolation of *Actinomycetes*

The sediment samples were collected using the Peterson grab at a depth of 5meter, from three stations in Parangipettai littoral zone (lat.11º 30', N; Long 76º 46' E) as well as in shrimp farms and fish ponds. Collected sediments were pretreated by keeping the sediments at 55ºC for six minutes in order to facilitate the isolation of *Actinomycetes.* About 5gm of the sediment sample was transferred to a flask containing 99ml of sterile sea water. Aliquots of the sediment suspension (0.5ml) were spread over the surface of agar media and incubated at room temperature for 2-3 weeks. The petri plates were observed from 5th day onwards for 3 weeks and the selected *Actinomycetes* were transferred to agar slants of the same isolation medium (Okazaki and Okami 1972)

Identification and characterization of the isolated *Actinomycetes*

Gram's staining

A loopful of overnight grown culture was taken and a uniform smear was made on a clear glass slide. It was heat fixed; crystal violet solution was added on to the slide and kept for a minute. The slide was then washed with water and flooded with gram's iodine solution. After a minute, the slide was counter stained with safranine and observed under the light microscope. The gram positive organisms appeared violet in color and gram negative organisms were pink in color (Shiriling and Gottileb 1966).

Starch hydrolysis

A loopful of culture was streaked on starch agar plates and incubated for 24 to 48h. The plates were flooded with Iodine solution. Absence of blue black color indicates the presence of starch and represents positive test.

Indole test

A loopful of culture was inoculated into the sterilized tryptone broth and an uninoculated tube was kept as control. The above tubes were incubated for 24 to 48h and 1 ml of Kovac's reagent was added to each tube including control. Development of cherry red color indicated a positive test for indole production.

Oxidase test

A loopful of culture grown overnight in the nutrient broth was applied over oxidase disc; changes in coloration from white to purple within 10 seconds indicates a positive reaction whereas no color formation denotes a negative oxidase test.

Hydrogen sulphide production

The culture was stabbed into SIM agar and after 24 to 48h of incubation black coloration along the stab indicates hydrogen sulphide production (a positive test).

Gelatin Liquefaction

Gelatin liquefaction have been studied by sub culturing the selected cultures on gelatin agar medium and using a heavy inoculums (growth from an 18 to 24 h pure culture) stab the tubes of gelatin agar with an inoculating needle directly down the center of the medium to a depth of approximately one half an inch from the bottom of the tube. The tubes, including an uninoculated control was incubated at 37ºC for 24 to 48h and up to 7days, observation was made after 7days. The extent of liquefaction was observed after keeping the tubes at cold condition (5-10ºC) for one hour.

Nitrate reduction

The pH of nitrate medium was adjusted to 7.2. Five ml volumes were dispensed into test tubes and autoclaved. The tubes were stab inoculated with 24 h grown culture. At intervals up to 5 days an aliquot of culture was withdrawn and tested for nitrite formation by adding a few drops of sulphanilic acid and α-naphthylamine reagent. A distinct pink or red colour indicated the presence of nitrite.

Utilization of various carbon sources

Carbon utilization test was performed using phenol red agar medium. Different carbon utilization discs were placed on phenol red agar swabbed with 24h culture of *Streptomyces*. The plates were incubated for 24-48h. After incubation the colour change was observed. The colour change to yellow indicates the utilization of carbon and absence of colour change indicates no carbon utilization.

Screening of antagonistic activity of isolated *Actinomycetes*

Antimicrobial activity of the bacterial strains was carried out following pour plate method. Briefly, bacterial plugs (6 mm diameter) of overnight culture were transferred to the surface of nutrient agar plates amended with bacterial pathogen (10^6 cfu/ml) and incubated at 28°C. The growth-free inhibition zones around the bacterial plugs were measured after 48 h incubation.

Extraction of secondary metabolites from potent *Actinomycetes*

The isolates PS2, PS4, PS9, PS11, PS13 and PS14 were screened for secondary metabolites production. For the large scale fermentation, overnight grown cultures of above isolates

were inoculated in 100 ml Nutrient broth at 37°C and from this 1% of the inoculum was made to 10 L of Nutrient broth and allowed for fermentation for 5 days at 37°C (Sambamurthy *et al*., 1974). The fermented culture was centrifuged at 10,000 *g* for 10 min in order to remove the cells and the supernatant (10 L) was extracted with equal volume of ethyl acetate from the strains. The organic layer was separated and concentrated under reduced pressure using rotary evaporator (Heidolph, Germany) and the crude extract was tested for broad-spectrum activity against human pathogenic.

Antimicrobial activity of crude ethyl acetate extracts of potent *Actinomycetes*

Crude ethyl acetate extracts of strains PS2, PS4, PS9, PS11, PS13 and PS14, were screened for their antimicrobial activity against human pathogenic bacteria *viz., Bacillus subtilis, Klebsiella pneumonia, Proteus vulgaris, Streptococcus faecalis, Pseudomonas spp.* by well diffusion method. The wells (6 mm) were impregnated with the 20µg/ml of crude metabolite (Katia *et al*., 2000) in MHA agar plate pre-inoculated with the test organisms. The above plates were incubated 37°C for 24 h and the diameter of zones of inhibition was measured.

RESULTS AND DISCUSSION

Isolation of *Actinomycetes*

Actinomycetes are gram positive bacteria well known for production of bioactive metabolites. Marine environment are significant as they comprises a larger part of soil at the bottom surface. Further, limited reports are available on the screening of microbial agents from marine soil sediment (Poopathi *et al*., 2013). In the present study 20 strains were isolated from the sediments of shrimp farms near Parangipettai. It was not possible to classify *Actinomycetes* colonies to the genus level while they are still in the primary culture. However all the strains where biochemically characterized using standard protocols (Table 1). All the 20 strains (PS1 to PS22) revealed positive response over H_2S production and Oxidase test. However none of the strains showed positive reaction for gelatin liquefaction test. Out of 20 strains of *Actinomycetes*, 14 strains were nitrate reduction test positive and negative for starch hydrolysis test. Out of 20 strains 17 were gram positive and 3 were gram negative. Similar trend was found in indole test. The carbohydrate utilization tests shows different pattern (Table 2). All the strains utilized dextrose and none of the strain utilized ducitol. Except one strain PS22 all the strains utilized the fructose and mannose. However only one Actinomycetes strain PS11 utilized inositol. Other carbohydrate sources like mannitol, xylose, galatose, and adonitol were utilized by PS15, PS13, PS7, PS5 strains respectively.

Table 2. Utilization of carbon source by isolated *Actinomycetes* strains

Strains	1	2	3	4	5	6	7
PS1	+	–	+	–	+	+	+
PS2	+	–	+	–	+	+	–
PS3	+	–	+	–	–	+	–
PS4	+	–	+	–	+	+	+
PS5	+	–	+	–	–	+	–
PS6	+	–	+	–	–	+	+
PS7	+	–	+	–	+	+	+
PS8	+	–	+	–	–	+	–
PS9	+	–	+	–	–	+	+
PS11	+	–	+	+	+	+	–
PS12	+	–	+	–	–	+	+
PS13	+	–	+	–	–	+	–
PS14	+	–	+	–	–	+	–
PS15	+	–	+	–	–	+	–
PS17	+	–	+	–	–	+	–
PS18	+	–	+	–	–	+	–
PS19	+	–	+	–	+	+	–
PS20	+	–	+	–	–	+	–
PS21	+	–	+	–	–	+	+
PS22	+	–	–	–	–	–	–

(–) Negative; (+) Positive; 1. Dextrose; 2. Ducitol; 3. Fructose; 4. Inositol; 5. Galactose; 6. Mannose; 7. Xylose

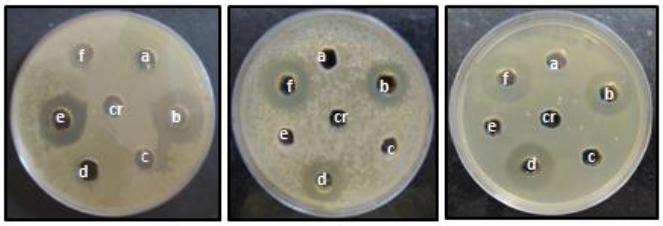
Table 1. Biochemical characterization of marine *Actinomycetes* isolated from marine sediments

Strains	Gram's Staining	Starch hydrolysis	Nitrate reduction	Gelatin liquefication	Hydrogen sulphide	Indole	Oxidase
PS1	+	–	–	–	+	–	+
PS2	+	–	+	–	+	–	+
PS3	+	+	+	–	+	–	+
PS4	+	–	–	–	+	–	+
PS5	+	+	+	–	+	–	+
PS6	–	–	+	–	+	–	+
PS7	–	–	+	–	+	–	+
PS8	+	+	+	–	+	+	+
PS9	+	–	–	–	+	–	+
PS11	+	+	+	–	+	–	+
PS12	+	–	+	–	+	+	+
PS13	+	–	+	–	+	–	+
PS14	+	+	–	–	+	–	+
PS15	+	–	+	–	+	–	+
PS17	+	–	+	–	+	–	+
PS18	+	–	–	–	+	–	+
PS19	+	+	–	–	+	+	+
PS20	+	–	+	–	+	+	+
PS21	–	–	+	–	+	–	+
PS22	+	–	+	–	+	–	+

(–) Negative; (+) Positive

Antibacterial activity of crude ethyl acetate extract of potent strains

Based on the antagonistic activity of the total isolates, six strains PS2, PS4, PS9, PS11, PS13 and PS14 were selected (Table 3) and cultured in nutrient broth supplemented with sea water and the compounds extracted with ethyl acetate. The extracts were tested against *Bacillus subtilis, Klebsiella pneumonia, Proteus vulgaris, Streptococcus faecalis, Pseudomonas spp.* The ethyl acetate extracts of all the six isolates inhibited three tested bacteria (Table 4). The maximum inhibition was observed against *Pseudomonas* spp followed by *Streptococcus faecalis, Bacillus subtilis,* respectively. Isolates PS4, PS11 and PS14 showed good inhibition of test bacteria.



(a) *Bacillus subtilis* (b) *Streptococcus faecalis* (c) *Pseudomonas spp.*

Fig. 1 Effect of crude ethyl acetate extracts of strains (a) PS2, (b) PS4, (c) PS9, (d) PS11, (e) PS13 and (f) PS14 on human pathogenic bacteria

Table 3. Antagonistic activity of *Actinomycetes* isolated from marine sediments

Strains	1	2	3	4	5
PS1	–	–	–	+	–
PS2	–	–	–	–	–
PS3	–	–	–	–	–
PS4	+	+	+	+	+
PS5	–	–	–	–	–
PS6	–	–	–	–	–
PS7	–	+	+	–	–
PS8	–	–	–	–	–
PS9	+	+	+	+	+
PS11	+	+	+	+	–
PS12	–	–	–	–	–
PS13	–	–	–	–	–
PS14	+	+	+	+	+
PS15	–	–	–	+	–
PS17	+	–	–	–	–
PS18	–	–	–	–	–
PS19	–	–	–	–	–
PS20	–	–	–	–	–
PS21	–	–	–	–	–
PS22	–	–	–	–	–

(–) Negative; (+) Positive; 1. *Bacillus subtilis;* 2. *Klebsiella pneumonia;* 3. *Proteus vulgaris;* 4. *Pseudomonas spp.;* 5. *Streptococcus faecalis*

Table 4. Effect of crude ethyl acetate extracts of *Actinomycetes* against human pathogens by well diffusion

Test organisms	Inhibition zone (mm)					
	PS2	PS4	PS9	PS11	PS13	PS14
Bacillus subtilis	0	16	0	0	0	17
Klebsiella pneumonia	0	0	0	0	0	0
Proteus vulgaris	0	0	0	0	0	0
Streptococcus faecalis	0	18	0	15	0	20
Pseudomonas spp.	0	18	0	22	0	22

Conclusion

Present study clearly indicated that the ethyl acetate extract of strain PS14 of *Actinomycetes* possesed good antimicrobial potential. Further future identification of strain at molecular level, purification of secondary metabolites and structural elucidation will be a fruitful outcome for betterment of mankind.

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