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Research Article

ISOLATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF ACTINOBACTERIA FROM POINT CALIMERE COASTAL REGION, EAST COAST OF INDIA

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ABSTRACT

The marine environment is a potential source of secondary metabolites, which provides sustainable source of supply for developing novel pharmaceutical leads. A total of 192 actinomycetes colonies were isolated from near shore marine environment of Point Calimere, East Coast of India. Among them, 68 isolates were morphologically distinct on the basis of spore mass colour, reverse side colour, aerial and substrate mycelia formation and production of diffusible pigment. Antimicrobial activities of isolates were also tested against various bacterial and fungal pathogens. Of 68 isolates, 25 (36.8%) isolates had antimicrobial activity, among 25 antimicrobial compound producers, 22 (88%) isolates could inhibit bacterial pathogens, 16 (64%) isolates was possessed antifungal activity, and 13 (52%) isolates inhibit both bacterial and fungal pathogens. The results of the present investigation revealed that, the marine actinomycetes from the Point Calimere, east coast of India are a potential source of novel antibiotic leads.

KEY WORDS: Point Calimere, East coast of India, actinomycetes, antimicrobial activity.

INTRODUCTION

Screening of microbial natural products continues to represent an important route to the discovery of novel chemicals for the development of new therapeutic agents and for evaluation of the potential of lesser-known and/or new bacterial taxa[1]. Among the microorganisms, actinomycetes gain special importance, as they are the most potent source for the production of antibiotics and other bioactive secondary metabolites. Actinomycetes are gram-positive bacteria, free living, saprophytic bacteria widely distributed in soil, water and colonizing plants showing marked chemical and morphological diversity but form a distinct evolutionary line of organisms[2]. Actinomycetes are potential source of many bioactive compounds[3-6] which have diverse clinical effects and important applications in human medicine[7]. It has been estimated that approximately one-third of the thousands of naturally occurring antibiotics have been obtained from actinomycetes[8]. Indeed, *Streptomyces* species produce about 75% of the commercially and medically useful antibiotics[9].

Most actinomycetes were believed to be terrestrial; however, some strains have also been found to occur in marine environments. Some actinomycetes were isolated from a marine environment which required seawater for growth and these strains were designated marine actinomycetes[10]. Oceans account for more than 70% of the earth's surface and the microorganisms growing in marine environments are metabolically and physiologically diverse from terrestrial organisms[8]. The prevalence of antimicrobial resistance among pathogens is increasing at an alarming rate worldwide[11]. Hence, the pharmaceutical

communities are in the position to find out the sources of antimicrobial compounds from various resources especially microbial origins. In the present study, an effort was made to isolate, evaluate the antimicrobial potentiality of actinomycetes from Point Calimere evergreen Reserve Forest, East Coast of India, with emphasis on the potent antibiotic producer's morphology, cultural, physiological and chemotaxonomic properties.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from evergreen reserve forest of Point Calimere, which located in East Coast of India (Lat. 10^{o} 18' N and Long. 79^{o} 51' E) Tamil Nadu. Seashore soil samples were collected at random in sterile polythene bags (brought to the laboratory) and stored for further use.

Isolation of actinomycetes

Starch casein agar (SCA) medium[12] (g/l: starch 10; casein 0.3; KNO_3 2; NaCl 2; K_2HPO_4 2; $MgSO_4$ $7H_2O$ 0.05; $CaCO_3$ 0.02; $FeSO_4$. $7H_2O$ 0.01; agar 18) was prepared and sterilized at 121^{o}C in 15 lbs pressure for 15 min and supplemented with amphotericin B (50 µg/ml) and tetracycline (20 µg/ml) (Himedia, Mumbai) to prevent the fungal and bacterial growth respectively. The collected soil samples were diluted up to 10^{-6} and 0.1 ml of the diluted samples were spread over the agar plates, and triplicates were maintained. The inoculated plates were incubated at 28±2^{o}C for seven to ten days. After incubation, the actinomycetes colonies were observed, purified and maintained in SCA medium for further investigation[13].

Screening of antimicrobial compounds producing actinomycetes

Initially, antimicrobial producing properties of the actinomycetes were screened by cross streak method[14]. Single streak of the actinomycetes was made on the surface of the modified nutrient agar medium (g/l: yeast extract 3; NaCl 5; Peptone 5; glucose 5; agar 15; pH 7.1) and incubated at 28±2^{o}C. After observing a good ribbon like growth of the actinomycetes on the plates, the bacterial pathogens namely *Escherichia coli* and *Bacillus subtilis,* and fungi *Cryptococcus neoformans* and *Candida albicans* were streaked at right angles to the original streak of actinomycetes and incubated at 37^{o}C for bacteria and 27^{o}C for fungus, the inhibition zone was measured after 24-48 h. Based on the presence and absence of inhibition zone, the antimicrobial compounds producing actinomycetes were selected.

Antimicrobial efficacy

The intense antimicrobial compound producing actinomycete strains were selected and its antimicrobial spectrum was tested against the pathogenic bacteria and fungi obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The selected isolates were inoculated into SC broth, and shaken at 28±2°C and 250 rpm for seven to ten days. After incubation, the staling substances were filtered through filter paper (Whatman No.1) and then through Seitz filter (G5). The filtrate was transferred aseptically into the conical flasks and stored at 4°C for further assay. An equal volume of five different solvents (alcohol, chloroform, distilled water, ethyl acetate and methanol) were separately added to the cell free culture filtrates and shaken for 2 h and the antimicrobial compounds were extracted by the methods of Gandhimathi *et al*.[15]. Antimicrobial activity of actinomycetes was determined by using well diffusion method[16,17]. Eleven different pathogenic bacteria were inoculated onto Muller Hinton agar and two fungal pathogens were inoculated in to Sabouraud's dextrose agar. After solidification, wells were made over the lawn cultures of bacteria and fungi using sterile well cutter (6 mm), and add 25 µl of solvent dissolved extracts separately. The plates were incubated at 37^{o}C for bacteria and 27^{o}C for fungi. After 24-48 h of incubation the diameter of the zone of inhibition was measured to evaluate the antimicrobial activity of actinomycetes isolates.

Characterization of actinomycetes

Light Microscopy: Morphological characteristics of the strain were observed by slide culture technique[18,19] after incubation of 7-10 days. After incubation the slides were observed under the light microscope (Leitz Diaplom, Germany).

Electron microscopy: Cells were grown for 5 to 10 days in cultivation broth shaken at 250 rpm and at 28±2°C. Cells were then fixed by adding 3.7% formalin and washed three times with two volumes of deionized water. Resuspended cells were spotted on a glass slide, flash frozen in a bath of 2-methylbutane in liquid nitrogen, freeze-dried, and then sputter coated with gold-palladium alloy under vacuum. Sample was visualized under Scanning Electron Microscope.

Biochemical, physiological and cultural characteristics: Biochemical characteristics of the strain were determined following the methods of International *Streptomyces* Project[20] (ISP). The production of hydrogen sulphide, triple sugar iron and, reduction of nitrate, liquefaction of gelatin, hydrolysis of casein, starch and lipid, and enzyme reactions such as, urease, catalase, oxidase and β-lactamase were determined. The ability to grow at various temperatures (10-40°C), range of pH (7-9) and in different concentrations of NaCl (2-16 g/l) on medium was also tested. The organism was also tested for its ability to utilization of carbon sources such as dextrose, fructose, glucose, inositol, lactose, maltose, mannitol, rhamnose, starch, sucrose and xylose in modified Bennett broth[21]. Cultural characteristics of the strain were determined following incubation for 10-15 days at 28±2°C on culture media recommended by the ISP and other culture media namely, yeast extract malt extract agar (ISP medium 2), inorganic salt starch agar (ISP medium 4), glycerol-asparagine agar (ISP medium 5), asparagine mannitol agar, Kenknight agar, nutrient agar, starch nitrate agar, SCA, yeast extract starch agar and potato dextrose agar (Himedia, Mumbai). After incubation the growth, color of spore mass, reverse side colour and diffusible pigment production were observed.

Chemotaxonomy: Isomers of diaminopimelic acid (DAP) in cell wall hydrolysates and whole cell sugars of actinomycete was determined by thin layer chromatography following the standard methods of Waksman and Henrici[22] and Boone and Pine[23].

RESULTS AND DISCUSSION

The filamentous bacteria, especially streptomycetes, are commonly found in almost all habitats. In the present study, totally 192 actinomycete colonies including white, ash, blue, green and brown coloured colonies with different morphological types were isolated from seashore soils, among 192 actinomycete colonies, 68 morphologically distinct actinomycetes were selected for further antimicrobial study. It has also been well established that most of the actinomycetes exhibit antimicrobial activity. It was found that 25 (36.8%) [>5 mm of inhibition zone produced isolates only considered as antagonist] isolates among 68 isolates of actinomycetes showed antimicrobial activity against various pathogens tested. Among 25 antimicrobial compound producers, 22 (88%) isolates could inhibit bacterial pathogens, 16 (64%) isolates was possessed antifungal activity, and 13 (52%) isolates inhibit both bacterial and fungal pathogens. Of 22 antibacterial compound producing actinomycetes, 15 (68%) isolates inhibited the growth of Gram-positive bacteria, 16 (72.7%) isolates had activity against Gram-negative bacteria and 9 (40.9%) isolates had activity against both Gram-positive and Gram-negative bacteria (Fig. 1). Ultimately, one isolate namely VPTS3-2 was selected for further study based on their potential antimicrobial activity against all the pathogens tested. The antimicrobial potentiality of the actinomycetes, especially *Streptomyces* are well known. However, there are both quantitative and qualitative variations in the antibiotics produced by different genera and species. Substrates and habitats greatly influenced the production of antibiotics by actinomycete isolates.

In the present study, the antimicrobial efficacy of the isolate VPTS3-2 was evaluated by using five different solvents namely alcohol, chloroform, aqueous, ethyl acetate and methanol. Among the solvents used, ethyl acetate extract was showed maximum antimicrobial activity, whereas the other solvent extracts showed moderate to minimum activity against all the pathogens tested. Comparatively, the ethyl acetate extract of the isolate had more antifungal activity than antibacterial activity. The ethyl acetate extract of the isolate VPTS3-2 showed maximum activity against *Cryptococcus neoformans* (19 mm), followed by *Candida albicans* and *Bacillus subtilis* (16 mm) and *E. coli* (15 mm) (Table 1). Similarly, various solvents were used for the extraction of antibiotics from actinomycetes by many workers using ethyl acetate, methanol, chloroform, n-butanol, n-hexane, petroleum ether, chloroform,

benzene and xylene. Hence the present study it has been recommended that the selection and cost of solvent for the extraction is also play vital step in the antimicrobial compound production in large scale level.

The results of the identification protocol of antagonistic isolate are shown in table 2 and 3. Morphological characteristic features such as, formation of ash coloured aerial spore mass, light blackish reverse side, no diffusible and melanin pigment producion, development of both aerial and substrate mycelium, development of hook like spore chains and smooth spore surface of the strain, and chemotaxonomic studies presence of L-DAP in cell wall and absence of characteristic sugars in their cell, which convincingly categorize the cell wall of the strain belonged to the cell wall type-I hence, the above characteristic features of the strain VPTS3-2 justifiably make to place the strain under the genus *Streptomyces* (Table 2).

The strain grew well at temperature at 30^{o}C, pH 8 and NaCl concentration 2-4 g/l. Cultural studies revealed that the strain VPTS3-2 grew well on several media including SCA, ISP 5 and ISP 7, developed whitish and ash coloured aerial mycelium and the reverse side of the media became white, brown and ash in most of the media tested (Table 2 and 3). Thus the biochemical, cultural and physiological characteristics such as tolerance to varied temperatures, pH and NaCl and utilization of carbon sources, confirm the identification of the strain as *Streptomyces* sp. VPTS3-2. Thus the present investigation concludes that the biochemical and physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and the physical conditions. It is evident that the growth of the actinomycetes was influenced by the environmental factors such as pH, temperature, inhibitory compounds and the availability of nutrients.

Conclusively, the search for novel antibiotics especially from marine actinobacteria needed a huge population of isolates in order to discover an actinomycete with novel compound of pharmaceutical interest. Because of this, the research will be more promising if diverse and more actinomycetes are isolated and screened. In this context, the present study was an attempt to identify and pick-out versatile strains of *Streptomyces* from the regions of the Point Calimere east coast of India that display antimicrobial activity against a variety of microbial pathogens intrinsically. Further studies on the purification and characterization of the antimicrobial secondary metabolites are currently in progress.

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Table 1: Antimicrobial efficacies of actinomycete isolate VPTS3-2

Name of the pathogens	Zone of inhibition (mm)				
	Alcohol	Chloroform	Distilled water	Ethyl acetate	Methanol
Bacillus subtilis	7	9	5	16	12
Escherichia coli	9	5	4	15	9
Candida albicans	9	10	10	16	6
Cryptococcus neoformans	10	5	6	19	7

Table 2: Morphological, cultural and biochemical properties of *Streptomyces* sp. VPTS3-1

Name of the test	Properties
Morphological properties	
Sporophore morphology	Spiral
Spore surface	Smooth
Colour of aerial mycelium	Ash
Colour of substrate mycelium	Light black
Spore mass	Ash
Gram staining	Gram positive
Acid fast staining	Non acid fast
Cultural properties	
Asparagine mannitol agar	Good
Yeast extract malt extract agar (ISP 2)	Moderate
Inorganic salt starch agar (ISP 4)	Poor
Glycerol asparagine agar (ISP 5)	Good
Kenknight agar	Poor
Nutrient agar	Excellent
Starch nitrate agar	Good
Starch casein agar	Excellent
Potato dextrose agar	Good
Yeast extract starch agar	Moderate
Biochemical properties	
H_2S production	+
Nitrate	-
Urease	+
Catalase	+
Oxidase	-
β-Lactamase	-
Melanin	-
Starch	+
Gelatin	-
Lipid	-
Casein	+
Haemolysis	-
Triple sugar iron	+
Chemotaxonomic properties	
Di-amino pimelic acid	+
Cell wall sugars	-

- = negative; + = positive

Table 3. Physiological, carbon and nitrogen source utilization tests of *Streptomyces* sp. VPTS3-1

Name of the test	Properties
Growth temperature (°C)	
10	-
20	+
30	+++
40	++
Growth pH	
5	-
6	+
7	+++
8	++
9	+
NaCl tolerance (% w/v)	
1	++
2	+++
4	+++
8	++
16	+
Carbon source utilization	
Dextrose	+
Fructose	+
Glucose	+
Inositol	+
Lactose	+
Maltose	+
Mannitol	+
Rhamnose	+
Starch	+
Sucrose	+
Xylose	+
Nitrogen source utilization	
Dl-alanine	+
L-arginine	+
L-cysteine	-
L-glycine	+
L-isoleucine	-
L-leucine	-
L-lysine	+
L-methionine	+
L-phenylalanine	+
L-proline	-
L-serine	+
L-threonine	+
DL-tryptophan	+
L-thyrosine	+

L-valine	-

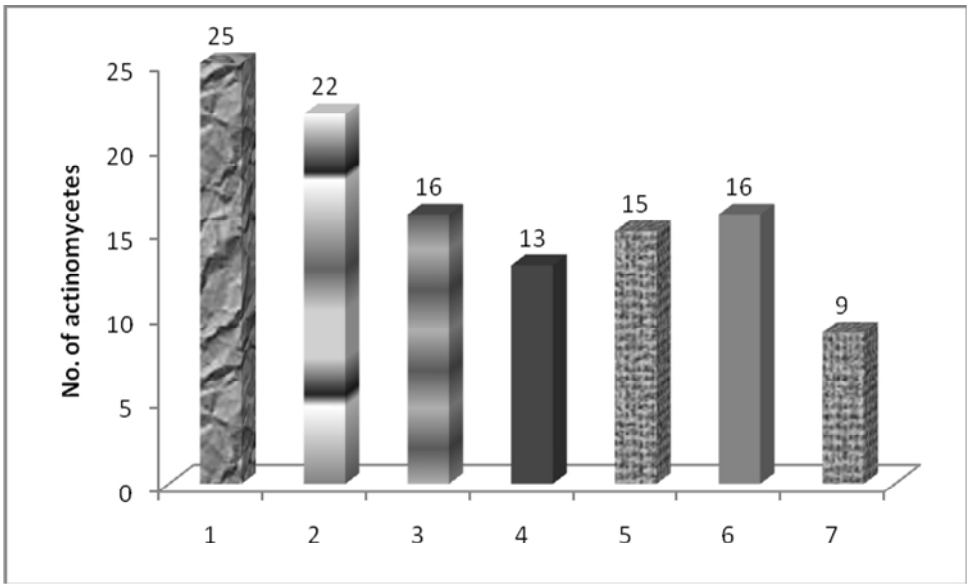

Fig. 1: Antimicrobial activity pattern of actinomycete isolates

1. Total antagonist; 2. Antibacterial; 3. Antifungal; 4. Both antibacterial & antifungal; 5. Against Gram+ve bacteria; 6. Against Gram-ve bacteria; 7. Both G+ve & G-ve bacteria

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