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Antibacterial activity and characterisation of Actinobacteria isolated from Marine Bivalve *Meretrix casta* (Gmelin)

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ABSTRACT

The purpose of this work was to screen the actinobacteria having antimicrobial activity of fourteen strains of Streptomycetes which was isolated from the marine bivalves Meretrix casts (Gmelin) Parangipettai coastal region. Four isolates showed a strong activity against S. aureus. These isolates where extensively studied for their in vitro anti-microbial activity against Gram-positive and Gram- negative bacteria. The results indicated that obtained isolates were highly active against S. aureus, E. coli, P. mirabilis and S. pyogenes in primary screening. The morphological, micromorphological, physiological and biochemical results obtained for the antibiotic producing strains were compared with those of the Streptomyces species given in the key of Nonomura (1974) and Berey's Manul of Determinative Bacteriology (Buchanan and Gibbons, !974) and was identified four strains as Streptomyces orientials (PS-1), S. hastedii (PS-2), S. nigellus (PS-3), and S. sclerotialus (PS-8). Hence it is suggested that the strains isolated and identified from the marine bivalve possessing antibiotic properties are worth investigating in details in future for isolating the antibiotics for human welfare.

Key Words: Mollusks, Actinomycetes, Human bacterial pathogens, antagonistic activity.

INTRODUCTION

Actinobacteria are gram-positive bacteria, which comprise a group of branching unicellular microorganisms. They produce branching mycelium, which may be of two kinds: substrate and aerial mycelium. They form a distinct group on the basis of nucleic acid sequencing and pairing studies and generally exhibit branched filaments, though some show pleomorphic and even coccoid elements. Despite this, actinobacteria are often regarded as the prokaryotic equivalent of fungi. They are widely distributed in soil, composts, water and other environment.

Actinobacteria have been looked upon as a potential source of bioactive compounds and the past experience proves that they are richest sources for the production of secondary metabolites. They hold a prominent position as targets in screening programmes due to their diversity and their proven ability to produce novel metabolites and other molecules of pharmaceutical interest (Ellaiah *et al.*, 2004). Since, the discovery of actinomycin (Lwchevalier, 1982), actinobacteria have been found to produce many commercially important bioactive compounds and antitumor agents in addition to enzymes of industrial interest. It is estimated that approximately two- third of thousands of naturally occurring antibiotics have been isolated from these microorganisms. The abundance of terrestrial actinobacteria and their productivity of metabolites are well known. The marine actinobacteria would be important sources for the discovery of new bioactive compounds.

MATERIALS AND METHODS

The molluscans samples were collected from the Parangipettai coastal area by hand picking and after collection, it was taken to the laboratory in sterile containers for isolation of actinobacteria. Before isolation of actinobacteria, the mollucan samples were thoroughly washed with sterile 50% sea water to remove the mud particles adhering to the shell surface. The shell surface was scraped with sterile scalpels; the scrapings were collected in sterile petridishes and then transferred aseptically to 100 ml of 50% sea water blanks previously sterilized. The animal was cut and opened in a sterile cabinet. A portion of the gut were removed and macerated with sterile acid-washed sand in a mortar and the transferred separately to sterile 50% sea water blanks. The flasks were thoroughly shaken in rotary shaker for 10 to15 min and serial dilutions were then prepared. 1 ml aliquots of appropriate dilutions were pipetted out into sterile petridishes and plated in the Kuster's agar medium (KUA). To prevent the fungal and bacterial contaminations, cycloheximide (100mg/l) and nalidixic acid (20 mg/l) were added to the medium (Kathiresan *et al.*, 2005). The petri plates were then incubated at 28 ± 2^0C and the colonies were observed from 5^{th} day onwards for 1 month (Sivakumar *et al.*, 2005a). Strains of actinomycetes were picked out and purified by repeated streaking on yeast extract-malt extract agar (ISP-2) medium. The pure cultures of the actinomycetes were transferred to ISP-2 slants and preserved at 4 ± 2^0C.

Screening of actinobacteria strains for antibacterial activity

Primary screening

Antibacterial activity of isolated actinobacteria against *Bacillus cereus* (MTCC 430), *Klebsiella pneumonia* (MTCC 432), *E. coli* (MTCC 452), *Proteus* mirabilis *(MTCC* 442), *S.pyogenes* (MTCC 451) and *Staphylococcus aureus* (MTCC 87). was studied. The antibacterial activity was tested, using the cross streak method (Waksman and Lechevalier, 1962). Single streak of the actinobacteria was made on the surface of the modified nutrient agar (Sivakumar *et al.*, 2005a) and incubated at room temperature (28 ± 2^0C). After observing a good ribbon-like growth of the actinobacteria on the petriplates, the pathogen was streaked at right angles to the original streak of the actinobacteria and incubated at 28 ± 2^0C. The inhibition zone was measured after 24 and 48 h. A control plate was maintained without inoculating the actinobacteria, to assess the normal growth of bacteria. From this screening, strains of potential antagonistic actinobacteria were selected.

Secondary screening

Cultural conditions

The actinobacterial strain was inoculated in the flask containing soya bean broth and the flask was incubated at room temperature (28± 2ºC) for 14 days with agitation.

Extraction of antibiotics

Analytical grades chloroform, cyclohexane, ethyl acetate, dichloro methane were used as solvents for extraction of antibiotics form the supernatant solution. One part of culture filtrate in each pH value was extracted thrice with each solvent at room temperature (28± 2 ºC). Solvent – filtrate mixture was shaken thoroughly for 15 min. in a separating funnel and allowed to settle for another 15 min. After extraction with solvents, the aqueous phase was tested for their antibiotic activity against the test organisms and all the aqueous phases did not show any activity. Solvent layer was separated and evaporated at 50± 2ºC under vaccume The residue were dissolved in 50% ethyl acetate.

As four solvents were used to extract the antibiotics from cultures filtrates adjusted to three different pH values, totally four partially purified extract were obtained for each culture.

Antimicrobial activities of these methanol extracts were tested as follows. The pathogen (s), against which the culture was active, was swabbed onto AIA plates. What's man No.1 filter paper discs of 13 mm diameter were dipped into each of the ethyl acetate extract and placed on the AIA plates seeded with the pathogens. Then plates were incubated overnight at 37 ºC and zone of inhibition was measured.

Taxonomic investigation of potential actinobacterial strains

Taxonomical studies of selected isolates of actinobacteria were carried out according to the methods recommended by collaboratory in the ISP (Shirling and Gottlieh, 1966).

Morphological characteristics

Aerial mass colour: The colour of the mature sporulating aerial mycelium was recorded in a simple way (White, grey, red, green, blue and violet). When the aerial mass colour fell between two colour series, both the colours were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then also, both the colour series were noted. The media used were Yeast Extract-Malt Extract Agar and Inorganic-Salt Starch Agar.

Melanoid pigments:

The grouping was made on the production of melanoid pigments (*i.e.* greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. The strains were grouped as melanoid pigment produced (+) and not produced (-). In a few cases, the production of melanoid pigments was delayed or weak, and therefore, it was not distinguishable. This is indicated as variable (V). This test was carried out on the media ISP-1 and ISP-7 (Appendix I), as recommended by International *Streptomyces* Project (Shirling and Gottlieb, 1966).

Reverse side pigments:

The strains were divided into two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely, distinctive (+) and not distinctive or none (-).

In case, a colour with low chroma such as pale yellow, olive or yellowish brown occurs, it was included in the latter group (-).

Soluble pigments:
The strains were divided into two groups by their ability to produce soluble pigments other than melanin: namely, produced (+) and not produced (-). The colour was recorded (red, orange, green, yellow, blue and violet).

Spore chain morphology:
The species belonging to the genus *Streptomyces* were divided into three sections (Shirling and Gottlieb, 1966), viz. rectiflexibiles (RF), retinaculiaperti (RA) and spiral (S).
Characteristics of the spore-bearing hyphae and spore chains were determined by using direct microscopic examination of the culture surface. Adequate magnification (400X) was used to establish the presence or absence of spore chains and to observe the nature of sporophores.

Spore morphological characters of the strains were studied by inoculating a loopful of one week old cultures into 1.5% agar medium contained in test tubes, at 37^0 C. The actinobacteria was suspended and thoroughly mixed in the semisolid agar medium and 1 or 2 drops of the medium were aseptically pipette on to a sterile glass slide. A drop of agar was spread well on the slide and allowed to solidify into a thin film so as to facilitate direct observation under microscope. The cultures were incubated at 28 ± 2^0 C and examined periodically for the formation of aerial mycelium, spore structure and spore morphology.

Physiological characteristics
Assimilation of carbon source:
The ability of different actinobacteria isolates in utilizing various carbon compounds as sources of energy was studied following the method recommended by ISP. Chemically pure carbon sources certified to be free of admixture with the other carbohydrate or contaminating materials were used. Carbon sources for this test are D- glucose (Positve control), L- arabinose, Sucrose, D-xylose, Inositol, D-mannitol, D-fructose and Raffinose. These corbon sources were sterilized by either sterilization without heat. The media and plates were prepared and inoculated as suggested by ISP. The plates were observed from 5th day onwards. The growth on a given carbon source was always compared with controls, growth on basal medium alone and growth on basal medium plus. The results were recorded as follows:

Utilization Positive (+):
When growth on tested carbon was significantly better than on the basal medium without carbon but somewhat less than on glucose.

Utilization Doubtful (±):
When growth on tested carbon was only slightly better than on the basal medium without carbon and significantly less than with glucose.

Utilization Negative (-):
When growth was similar to or less than growth of basal medium without carbon.

RESULTS AND DISCUSSION

Population density of actinobacteria

During the present investigation, a total of 14 actinobacterial strains were isolated from the *Meretrix casta* sample and its population density was $1.4x10^4$ CFU/g.

Antagonistic activity

Primary screening

In the course of screening for novel antibiotics, ten actinobacterial strains were isolated from marine bivalves, collected near Porto Novo of south-east coast of the Bay of Bengal. All the ten actinobacteria subjected for primary process, of the ten isolates, all are active against Gram-positive and Gram-negative bacteria. Among them, 80% strains are against *S. aureus*, 70% are active against *K. pneumonia, E.coli, S. pyogenes and 60% are active against B. cereus, P. mirabilis* species (Table 1).

Table 1. Showing the primary screening for antagonistic actinobacteria against human bacterial pathogens

Activity against clinical pathogens (inhibition zone in mm)						
Isolates	***K. pneumonia***	***B. cereusi***	***E. coli***	***P. mirabilis***	***S. pyogenes***	***S. aureus***
PS-1	16	-	20	17	20	17
PS-2	17	19	19	16	16	23
PS-3	17	19	16	17	17	20
PS-4	-	-	16	-	16	14
PS-5	18	15	-	15	-	-
PS-6	-	17	20	15	16	17
PS-7	16	-	15	18	20	21
PS-8	16	19	16	-	16	14
PS-9	-	-	-	-	-	-
PS-10	20	19	-	18	-	18

- *denotes nil or less activity (inhibition zone below 15 mm)*

Secondary screening

Out of four potential strains in primary screening, only two strains were subjected for the secondary screening.

Table 2. Showing the secondary screening for actinobacteria against human bacterial pathogens

	Activity against clinical pathogens (inhibition zone in mm)							
Isolates	**PS-2**				**PS-3**			
Solvent Bacteria	**C**	**D**	**E**	**H**	**C**	**D**	**E**	**H**
K. pneumonia	-	18	-	-	18	-	20	-
S. boddi	-	16	19	-	15	17	15	-
E. coli	15	17	20	-	20	-	20	-
P. mirabilis	-	-	15	-	-	15	-	-
S. pyogenes	-	-	17	15	-	-	17	-
S. aureus	18	17	20	-	15	17	19	-

C- Chloroform, D- Di chloromethane, E- Ethyl acetate, n- hexane. – denotes nil or less than 15mm

Strains PS-2 and PS-3 were extracted with different solvents like chloroform, dichloromethane, ethyl acetate and n-hexane. Among the four solvents, ethyl acetate had good capacity of observing secondary metabolites from the culture broth. While comparing among the four, ethyl acetate showed 83.3% activity against tested pathogens, Di-chloromethane showed only 66.6% activity. Chloroform showed 33.3% activity against to the tested pathogens and Hexane showed no activity against any tested pathogens (Table 2).

Taxonomic investigation of the potential actinobacterial strains

In the present study, an attempt has been made to identify four isolates of actinobacteria to the species level. Description and distinctive characters of these four isolates are given in details. After locating closely related species, the description of the strains isolated in the present study we compared with the type cultures given in the ISP and identified.

Results of the analysis of cell wall components of the strains PS-1, PS-2, PS-3 and PS-8 are shown in Table 3. The strains PS-1, PS-2, PS-3 and PS-8 possesses LL-DAP and contains glycine in its cell wall. Presence of LL-DAP along with glycine indicates the cell wall chemotype I (Lechevalier and Lechevalier, 1970).

Table 3. Cell wall analysis of the four actinobacterial strains

Strain No.	LL-DAP	Meso-DAP	Glysine	Whole cell Sugars	Wall type
PS-1	+	-	+	-	**I**
PS-2	+	-	+	-	**I**
PS-3	+	-	+	-	**I**
PS-8	+	-	+	-	**I**

+ denotes presence; - denotes absence

Tables 4. Morphological and micromorphological characteristics of four actinobacterial strains.

Table 4.1 Comparison of morphological characteristics of strain PS-1 and *S. orientalis*.
Taxonomic investigation of the potential actinobacterial strains

Characteristics	Strain PS-2	*S. hastedii*
Colour of aerial mycelium	Grey	Grey
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	-	-
Sporechain morphology	RF	RF
Spore surface	smooth	Smooth
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	_
Mannitol	+	±
Fructose	+	+
Rhamnose	+	_
Sucrose	_	_
Raffinose	_	_

The genera belonging to the wall type - I are *Streptomyces, Streptoverticillium, Chainia, Actinopycnidium, Actinosporangium, Elyptrosporangium, Microellobosporia, Sporichthya* and *Intrasporangium* (Lechevalier and Lechevalier, 1970). The micromorphological observations of the strain PS-1, PS-2, PS-3 and PS-8 reveal that the strain belongs to the genus *Streptomyces*. Similar work reported that the strains AV-05, AV-11. AV-19, AV-24 and AV-30 showed good activity against pathogens and they were selected for identification

Table 4.2 Comparison of morphological characteristics of strain PS-2 and *Streptomyces halstedii*

CHARACTERISTICS	**Strain PS-1**	***S. orientials***
Colour of aerial mycelium	white	White
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	-	-
Sporechain morphology	RF	RF
Spore surface	smooth	Smooth
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Mannitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	±
Raffinose	+	±

Table 4.1 reveals that the strain PS-1 shows similar physiological and biochemical characteristics features when compared to the reference strain, S. *orientalis* except the strong utilization of sucrose and raffinose. Hence, the strain PS-1 has been tentatively identified as *Streptomyces orientalis.*

Table 4.3 Comparison of morphological characteristics of strain PS-3 and *Streptomyces nigellus*

CHARACTERISTICS	**Strain PS-3**	***S. nigellus***
Colour of aerial mycelium	Grey	Grey
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	-	-
Sporechain morphology	Spiral	Spiral
Spore surface	smooth	Smooth
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Mannitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	+
Raffinose	+	+

Table 4.2 shows that the strain PS-2 tested differed from the reference strain, S. *halstedii* by utilizing the carbon compound viz. inositol, rhamnose sugars and by strongly utilizing mannitol. However, the strain PS-2 resembles the reference strain by showing close similarity in all the other characters and hence the strain PS-2 has been tentatively identified as *S. halstedii*

Table 4.3 reveals that the strain PS-3 shows similar physiological and biochemical characteristics features when compared to the reference strain, S**.** *nigellus* . Hence, the strain PS-3 has been tentatively identified as *S* **.** *nigellus.*

Table 4.4 Comparison of morphological characteristics of strain PS-8 and *S. sclerotialus*

CHARACTERISTICS	Strain PS-8	*S. sclerotialus*
Colour of aerial mycelium	white	White
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	-	-
Sporechain morphology	Spiral	Spiral
Spore surface	Smooth	Smooth
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Mannitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	+
Raffinose	+	+

Table 4.4 reveals that the strain PS-8 shows similar physiological and biochemical characteristics features when compared to the reference strain, *S. sclerotialus*. Hence, the strain PS-8 has been tentatively identified as *S. scleroticalus.*

The morphological, micromorphological, physiological and biochemical characteristics of antagonistic strains (PS-1, PS-2, PS-3 and PS-8) tested in the present study, are given in the Tables 4.1to and 4. These characteristics were compared with those of the *Streptomyces* species given in the key of Nonomura (1974) and those species described in the Bergey's manul of Determinative Bacteriology (Buchanan &Gibbons, 1974).

Similarly the same work reported that strain AV- 19 is week in utilization of the carbon compounds *viz.* arabinose and xylose except this, all the other properties are the same for AV-19 and *S. violaceus.* Therefore, the strain AV-19 had been tentatively identified as *Streptomyces violaceus.* Similarly, the strain AV-24 is weak in the production of soluble pigment. Except this, all the other characters of the strain AV- 24 are exactly similar to that of *S. moderatus.* Therefore, the strain *S.orientalis* (PS-1), *S. hatsledii* (PS-2), *S. nigellus* (PS-3) and *S.sclerotialus* (PS-8) are tentatively identified.

CONCLUSION

The present study indicates that mollusc, *M. casta* (Gmelin) are a potential candidate for screening of antibiotic producing actinobacteria. Studies have been continued to purify and characterize the antibacterial compounds from the antagonistic marine actinobacteria and to test their possible use as alternative chemotherapeutic drugs and produce them on a commercial scale. If it is new, then it could be patented and commercialized.

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