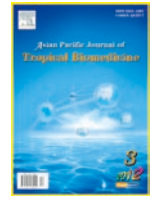




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In-vitro antimicrobial activity of marine actinobacteria against multidrug resistance *Staphylococcus aureus*

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ABSTRACT

Objective: To investigate the antibacterial activity of marine actinobacteria against Multidrug resistance *Staphylococcus aureus* (MDRSA). **Methods:** Fifty one actinobacterial strains were isolated from salt pans soil, costal area in Kothapattanam, Ongole, Andhra Pradesh. Primary screening was done using cross–streak method against MDRSA. The bioactive compounds are extracted from efficient actinobacteria using solvent extraction. The antimicrobial activity of crude and solvent extracts was performed using Kirby–Bauer method. MIC for ethyl acetate extract was determined by modified agar well diffusion method. The potent actinobacteria are identified using Nonomura key, Shirling and Gottlieb 1966 with Bergey's manual of Determinative Bacteriology. **Results:** Among the fifty one isolates screened for antibacterial activity, SRB25 were found efficient against MDRSA. The ethyl acetate extracts showed high inhibition against test organism. MIC test was performed with the ethyl acetate extract against MDRSA and found to be 1000 µg/ml. The isolated actinobacteria are identified as *Streptomyces* sp with the help of Nonomura key. **Conclusion:** The current investigation reveals that the marine actinobacteria from salt pan environment can be able to produce new drug molecules against drug resistant microorganisms.

1. Introduction

Multi drug resistance in microorganism is an emerging serious problem in health care sector. The improper usage of antibiotics contributes a major role for drug resistance in pathogenic microbes. Microorganisms acquire resistance towards common antibiotics by altering their metabolism and genetic structure [1, 2]. There is an incessant need to find novel efficient drug molecules against multi drug resistant microbes.

Staphylococcus aureus is a virulent pathogen that is responsible for a various infectious disease including sore throat, pneumonia, osteomyelitis, endocarditis, pimples and bacteremia. *S. aureus* has been reported resistance towards Methicillin and Vancomycin [3]. Methicillin resistance *S. aureus* (MRSA) was first reported in Britain by 1961 and was later found prevalent in hospitals. MRSA has been identified as an important nosocomial infection causing

organism [4]. Due to the development of drug resistance in *S. aureus*, treatment of this bacterial infection has become a serious problem [5]. Recently, new antimicrobial agents like daptomycin, linezolid and streptogramin combination (quinupristin/dalfopristin) are useful to treat MRSA [6, 7]. However, due to certain defects and escalation of drug resistance emphasize the requirement of advance antimicrobial agents having potential activity towards Gram positive bacteria[8].

Natural products are boundless source for important novel compounds having antagonistic activity against pathogenic organisms. Marine environment covers almost 70% of the earth surface [9]. Organisms present in these environments are extremely rich sources of bioactive compounds [10, 11]. The ocean remains as an unexploited source for many drugs and pharmacologically active substances [12]. Actinobacteria are Gram positive, filamentous bacteria which are supreme secondary metabolite producers [13]. Among the members of actinomycetes, the *Streptomyces* genus are a dynamic producer of functional, bio–effective metabolites with broad pharmaceutical range having antimicrobial, antihelminthic, antitumor and antiviral agents [14, 15].

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Actinobacteria from terrestrial origin produce hundreds of antibiotics which are widely used at present. Some differences could be expected among organisms existing in marine and terrestrial environments due to variation in the physical, chemical and biological factors [16]. It is apparent that the marine environment is a potent source for finding new actinobacteria and new antibiotics or biologically active substances [17]. The present work was undertaken to isolate potent actinobacteria from salt pan soil sample to elucidate their antimicrobial activity against multi drug resistance *S. aureus* (MDRSA).

2. Materials and Methods

2.1. Sample Collection

Soil samples were collected from salt pans, costal area at Kothapattanam, Ongole, Andhra Pradesh (15°30' 0" N, 80°3' 0" E), India during December 2011. Soil samples were collected at the depth of 10–25 cm. Samples were collected in sterilized container and transferred to the laboratory and stored in refrigerator at 4°C until further processing.

2.2. Isolation of Actinobacteria

Isolation and enumeration of actinobacteria were performed on selective media such as Actinomycetes Isolation Agar (AIA), Kuster's agars and Starch Caesin agar. The soil samples were serially diluted up to 10⁻⁷ and one milliliter of the serially diluted samples were inoculated into media. All these media are supplemented with cyclohexamide (100 µg/ml) to avoid fungal contamination. Inoculated plates were incubated at room temperature for 7 days [18].

2.3. Test organism

The clinical isolate of multi drug resistance *Staphylococcus aureus* was collected from Narayani Hospital, Ariyur, Vellore District, Tamil Nadu, India. The Test organism was maintained in glycerol stock and stored at -20°C.

2.4. Antibiogram

The multi drug resistant *S. aureus* was screened for their sensitivity towards standard antibiotics included, Ampicillin (10mcg/disc), Methicillin (10mcg/disc), Vancomycin (30 mcg/disc), Penicillin (10U/disc), Chloramphenicol (30mcg/disc), Drug sensitivity test was performed by disc diffusion method on Mueller Hinton agar (MHA) plates. Bacterial test pathogen was prepared inoculating into nutrient broth overnight. Isolates were lawn cultured on Mueller Hinton agar plates using sterile cotton swabs. The standard antibiotics discs were placed on the agar surface using a sterile forceps. Plates were incubated at 37°C for 24 hours and were observed for zone of inhibition [19].

2.5. Antimicrobial activity of actinobacterial isolates

2.5.1. Primary Screening of actinobacteria for antibacterial activity against MDRSA by– cross streak method

Primary screening of actinobacterial isolates were performed by cross streak method on modified nutrient agar plates (MNA) [20]. The actinobacterial isolates were inoculated in straight line on MNA plates and incubated for 7 days. MDRSA strain were cross streak on the same plate in perpendicular manner. The plates were incubated at 37°C for 24 hours. The plates were examined for the zone of inhibition of the MDRSA organisms.

2.5.2. Fermentation Process

The potent actinobacterial isolate were inoculated into production broth (SS Media) containing soluble starch–25g, glucose–10g, yeast extract–2g, CaCO₃ 3g, trace elements–1ml, Distilled water –1000ml. Flasks were lodged on the flask shaker at a speed of 120 rpm at room temperature for 7 days. After fermentation, the medium was harvested and centrifuged to remove cell debris. Filtrate was collected and lyophilized stored at 4°C for further use [21].

2.5.3. Secondary screening (Agar well diffusion method)

Secondary antimicrobial screening of actinobacteria was detected by agar well diffusion method on Mueller Hinton agar [22]. Multi drug resistance *S. aureus* was inoculated in nutrient broth and incubated for 24 hours at 37°C. The turbidity of the broth was adjusted at 0.5 (optical density) using spectrophotometer. The bacterial cultures were inoculated on MHA plates using sterilized cotton swabs. In each of these plates, wells were cut out using a sterilized gel borer. The crude and solvent extracts are used against test pathogen, 100 µl of extracts were loaded into each well. Plates were incubated at 37°C for 24 hours. After incubation, all plates were examined for the presence of zone of inhibition around the Wells [23].

2.5.4. Extraction of Bioactive Compounds

The bioactive metabolites were recovered from the harvested medium by solvent extraction method. The filtrate was mixed with ethyl acetate, chloroform, butanol (1:1 v/v) and shaken vigorously for 1 hour in a solvent extraction funnel. Solvent and filtrate mixture were stabilized for 24–48 hrs. After 48 hrs the solvent phase are separated from aqueous phase. The solvent extracts were concentrated and used for antibacterial activity [24, 25].

2.5.5. Determination of minimum inhibitory concentration

The MIC for ethyl acetate extract was determined by modified agar well diffusion method [26]. The concentration of test cultures was adjusted to 0.5 McFarland standards by using a spectrophotometer. Test organism was lawn cultured on the Muller Hinton Agar plates. Agar surface was bored by using a sterilize cork borer of 7 mm diameter. The extract is dissolved into ethyl acetate to obtain a concentration range from 25 to 1000 µg. A 100 µl of extracts in different

concentration were loaded in to wells. All test plates were incubated at 37°C for 24 hours.

2.6. Thin layer chromatography

Silica gel plates, 10x20 cm, 1mm thick, were prepared. They were activated at 150°C for half an hour. Ten micro liters of the ethyl acetate fractions were applied on the plate and the chromatogram was developed using chloroform: methanol (4:1) as solvent system. The spots in the chromatogram were visualized in the iodine vapour chamber and UV chamber [27].

2.7. Taxonomic Exploration

The efficacious actinobacteria are characterized by morphological and biochemical method and the results were compared with Nonomura key 1974, Shirling and Gottlieb 1966 and with Bergey's manual of Determinative Bacteriology [28]

2.7.1. Morphological characteristics

Actinobacteria isolate were inoculated in seven different International *Streptomyces* Project mediums (ISP 1 to ISP 7) and incubated for 7 days at room temperature. The colonies were observed under a high power magnifying lens and colony morphology was noted with respect to aerial mycelium color, nature of colony and reverse side color.

2.7.2. Assimilation of Carbon Source

The ability of different actinobacteria species in utilizing various carbon sources is analyzed. viz., arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose as sources of energy were studied based on the method recommended by International *Streptomyces* Project (ISP). These carbon molecules are sterilized by ether sterilization [29].

2.7.3. Generic investigation

The genus of actinobacteria with good antagonistic activity against the multi drug resistant *S. aureus* was identified using by cell wall composition analysis (amino acids and

whole cell sugars analysis) [30].

2.7.4. Statistical Analysis

All tests were conducted in triplicate. Data are reported as means±standard deviation (SD). Results were analyzed statically by using Microsoft Excel 2007 (Roselle, IL, USA).

3. Results

3.1. Antibiogram

S. aureus was screened for antibiogram by disc diffusion method on MH agar plates. The result exhibits that tested drugs did not showed any zone of inhibition against the *S. aureus*.

3.2. Isolation of actinobacteria

Soil samples were collected from salt pans near costal area in Kothapattanam, Ongole, Andhra Pradesh, India. The diversity of actinobacteria population was represented in figure–1. A total 51 actinobacteria colonies were isolated based on colony morphology and microscopic appearance (Table–1). The Actinomycetes Isolation agar enhanced more actinobacteria colonies, when compare to other media. The isolated strains were designated as SRB1–SRB51.

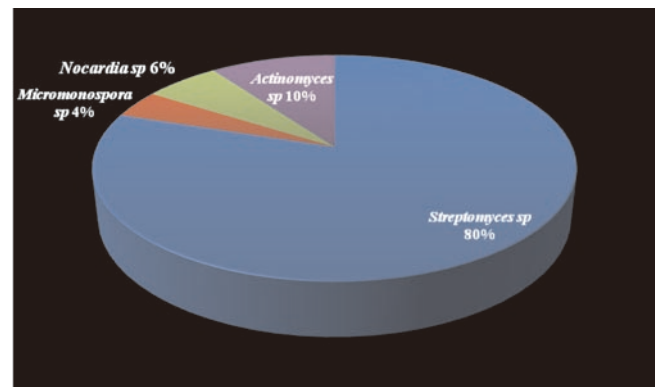


Figure 1. The diversity of actinobacteria population in marine salt pan soil

Table 1

Isolation of Actinomycetes using different media

Medium	No. of Plates Inoculated	Total no. of Actinomycetes Isolated	No. of Actinomycetes Recovered
Actinomycetes Isolation Agar	18	135	27
Starch Casein Agar	18	76	11
Kusters Agar	18	92	13

Table 2

Primary screening (Cross Streak Method)

Organism	Zone of inhibition (mm)				
	SRB1	SRB20	SRB25	SRB32	SRB38
MDRSA	6.4±0.25	5.1± 0.4	18.16±0.35	4.7± 0.4	6.2± 0.3

MDRSA: Multidrug resistant *S. aureus*

All values represent the mean±standard deviation

3.3. Antibacterial activity of isolated actinobacteria

Actinobacteria isolates were screened for antimicrobial activity against MDRSA. Among 51 actinobacteria isolates only five isolates (SRB1, SRB20, SRB25, SRB32 and SRB38) were showed activity towards test organism in cross streak method (Table 2). Out of 5 strains, SRB25 showed good antibacterial activity in agar well diffusion method. The potential strain SRB25 was inoculated into production medium (SS medium), the bioactive compound was extracted in different polarity solvents and the extracts were screened for antimicrobial activity against multi drug resistant *S. aureus*. The ethyl acetate extract exhibit potent activity against test organism, other solvent extracts did not showed any activity against the bacteria (Table 3). MIC test was performed with the extract against MDRSA and found to be 1000 μ g/ml (Fig – 2)

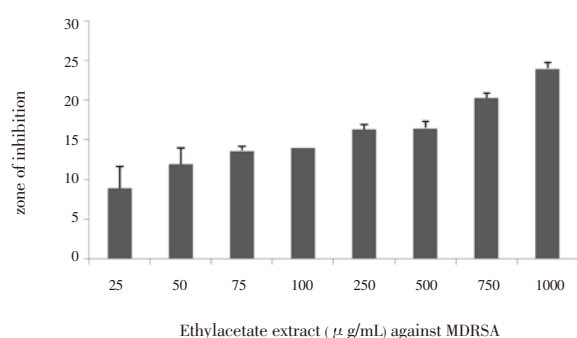


Figure 2. Minimum inhibitory concentration of SRB25 against MDRSA

3.4. Thin Layer Chromatography

The ethyl acetate extraction of SRB25 strain was carried out by thin layer chromatography using solvent system composed of chloroform: methanol (4:1). Total three different circular spots were obtained from the extract with a Rf values of 0.81, 0.54 and 0.37. The fluorescence compounds were observed under UV light. The compound showing fluorescence was partially identified as Actinomycin-D (Rf = 0.38).

Table 3

Secondary screening of isolate SRB-25 (Well diffusion method)

Test organism	Zone of Inhibition (mm)			
	CE	EE	BE	CE
MDRSA	23.33 \pm 1.15	24.66 \pm 0.57	n.a.	n.a.

CE: Crude extract, EE: Ethyl acetate Extract, BE: Butanol Extract, CE: Chloroform extract

n.a: No activity

3.5. Identification of Efficient Actinobacteria

The strain SRB25 possesses LL-DAP and contains glycine in its cell wall. Presence of LL-DAP along with glycine

indicates the cell wall chemotype I. The cell wall analysis of the strain SRB25 reveals that the strain belongs to the genus *Streptomyces*. The physiological characteristic of the strain SRB25 was compared with those of the *Streptomyces* species given in the key of Nonomura. The strain SRB25 showed more similarity when compared to the reference strain, *Streptomyces parvulus* (Table 4).

Table 4

Comparison of morphological characteristics of strain SRB25 and *Streptomyces parvulus*

Characteristics	Strain SRB25	<i>S. parvulus</i>
Colour of aerial mycelium	Grey	Grey
Melanoid pigment	–	–
Reverse side pigment	–	–
Soluble pigment	–	–
Sporechain morphology	Spiral	Spiral
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Mannitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	+
Raffinose	+	+

4. Discussion

Natural compounds obtain from marine source plays important key to discover various new drug molecules [31, 32]. Actinobacteria are the most potent and industrially important microorganism which are capable for the synthesis of bioactive compounds like enzymes, hormones, vitamins and other secondary metabolites. These bioactive compounds are highly difficult to synthesize artificially. Hence, these microbial compounds are considered to use as new drugs [33, 34, 35]. Marine microorganism are entirely varies from terrestrial microbes. The marine actinobacteria producing bioactive compounds are varying from terrestrial actinobacteria. Most of the 70% commercial antibiotics are obtained from soil actinobacteria [36].

Multi drug resistant *S. aureus* serves as a hospital borne pathogen and plays a dominant role in many clinical problems globally [37]. In Indian hospitals, MRSA is one of the common causes of hospital acquired infections and different hospitals reported about 30% to 80% methicillin resistance. The organism has the ability to cause several outbreaks in hospitals. The choice of drugs to be treating against MRSA is very less due to their genetic alteration, enzyme variation and permeability changes. Due to harmful effects, there is a need to find out new drug molecules against MDRSA [38].

In 2011, karthik et al reported marine sediments were good sources for isolation of actinobacteria and M2 media good for isolation of marine actinobacteria. Supporting to that our study also revealed that marine salt pan soil harbored more actinobacteria [39]. Baskaran et al reports that starch casein agar, Kuster's agar was found to be good for isolation of marine actinobacteria population. However,

in the present study the maximum number of colonies were isolated on AIA followed by kuster's agar [40]. Hence, the soil physicochemical properties may play a major role in the selection of isolation media.

The 51 isolates which are isolated from marine salt pan soil were subjected to primary screening, among them only five strains showed anti bacterial activity. As the strain SRB25 showed maximum inhibition zone, it was selected for secondary screening. Pickup et al reported that among the 134 actinobacteria isolates were subjected to antimicrobial activity, 51 isolates showed good activity in primary screening but failed to manifest activity in secondary screening [41]. The *Streptomyces* sp isolated from saline farmlands possess both antibacterial and antifungal activity [42]. The *Streptomyces* sp PM-32 isolated from offshore sediments collected at the Bay of Bengal coast has been shown to possess antimicrobial activity against a group of bacterial and fungal pathogens [43].

The bioactive compounds are extracted from natural sources through several techniques. Solvent extraction is usually employed for the extraction of secondary metabolites from the culture filtrates. Different polarities of organic solvents have been utilized for the extraction of bioactive compounds from actinobacteria [44]. The extracts from Ethyl acetate showed maximum antimicrobial activity against bacteria and fungi, other solvents extracts showed moderate activity against test organism [45]. Same trend was observed in this study and also found that Ethyl acetate solvent was most appropriate for compound extraction. The ethyl acetate extract of SRB25 showed potent activity against MDRSA. The minimum inhibitory concentration (MIC) for the antimicrobial extracted from SRB25 was 1000 µg/ml. According to the TLC separation, the three fluorescence spots were detected under UV radiation. The ethyl acetate extract yielded components showed Rf value 0.37 which are similar to Rf value of Actinomycin-D [46]. The Actinomycin-D acts as potent antineoplastic drug molecules produced by *Streptomyces parvulus* used to inhibit cell proliferation in tumor cells. [47]. This molecule may be responsible for inhibition of growth of MDRSA. Based on the chemotaxonomy, the genera belonging to the cell wall type - I are *Streptomyces*, *Streptoverticillium*, *Chainia*, *Actinopycnidium*, *Actinosporangium*, *Elyptosporangium*, *Microellobosporia*, *Sporichthya* and *Intrasporangium* [48]. The morphological observations and cell wall analysis of SRB25 reveals that these isolate belong to the genus *Streptomyces*. The sugar utilization test and other characters are exactly similar to those of *Streptomyces parvulus*. Hence, the strain SRB25 has been identified as *S. parvulus*. The antibiotic producing *Streptomyces* which are isolated from mangrove environment are identified with the help of Nonomura key (1974) and those species described in the Bergey's Manual of Determinative Bacteriology [49]. Based on the importance of marine actinobacteria, Current studies were focused to detect the bioactive compounds from marine actinobacteria of unexplored salt pan environment. Actinobacteria were isolated from salt pan soil samples which are showing high tremendous activity against drug resistant strain. The identification and production of new drug molecules from marine actinobacteria are necessary to counteract drug resistance in microbial population. Further investigations are needed in order to determine the active metabolites.

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Conflict of interest statement

We declare that we have no conflict of interest.

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