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ANTIBACTERIAL ACTIVITY OF MARINE *STREPTOMYCES* SP. ISOLATED FROM ANDAMAN & NICOBAR ISLANDS, INDIA

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ABSTRACT

Andaman and Nicobar Islands, considered as one of the important marine ecosystem in India and being considered as the richest source for microbial community. The marine microbial taxa and actinomycetes diversity are not been fully explored. Actinomycetes were isolated from marine soil samples and screened for antibacterial activity. Out of eight isolates, the isolate AN1 isolated from soil sample collected from Elephanta beach showed significant antibacterial activity against ATCC strains *Staphylococcus aureus* (19 mm), *Bacillus cereus* (17 mm) and *Salmonella paratyphi* A (16.5 mm). The ethyl acetate (EA) extract of the isolate showed increased activity against *Staphylococcus aureus* (22.1 mm), *Bacillus cereus* (19.5 mm) and *Salmonella paratyphi* A (19.6 mm). The standard antibiotic streptomycin disc (25µg) was used as positive control. The isolate AN1 were subjected to biochemical characterization and identified as *Streptomyces* species. The result of this study suggests that the *Streptomyces* species could be a promising source for potent antibacterial agents.

KEYWORDS: **Andaman and Nicobar Islands; marine ecosystem; actinomycetes; antibacterial activity.**

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INTRODUCTION

Marine microbial community are vast and remains to be a richest resource for bioactive compounds which are untapped by the marine biologist. The critical marine environments are the reason for the production of secondary metabolite by these microbial groups adding more significance than terrestrial environment. The hunts for potent compound from the marine ecosystem for treatment of various drug resistant pathogenic diseases are still in progress even though several compounds successfully marketed. Among the micro organisms, actinomycetes are well known marine fungi like bacterial group, ubiquitous in nature, produces major antibiotics accounts for 70% of the world wide antibiotics especially produced by *Streptomyces* species[1]. The extreme environment of salinity and pressure exist in the marine environment cause these actinomycetes to adapt and produce natural compounds[2]. Researchers have already isolated more than 11,000 marine derived natural products [3] and several compounds have been shown to posses significant bactericidal activity [2]. A novel antibiotic fridamycin extracted from marine *Streptomyces fradiae* showed potent antimicrobial activity, Xin et al., 2012[4]. Lactonamycin produced by marine *Streptomyces risfiiriensi*s exhibited significant antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) [5]. Fielder et al., 2005[6] screened 600 actinomycetes strains isolated from marine sediments of Pacific and Atlantic Oceans for the production of bioactive secondary metabolites and reported that marine *Streptomyces* are well known for producing chemically diverse antibiotics. The marine ecosystem of Andaman and Nicobar Islands provided a novel and efficient antimicrobial compounds[2]. Microbial diversity and antimicrobial activity of isolates from great Nicobar Islands and Andaman Archipegales has already been reported[7]. The marine ecosystem of Andaman and Nicobar Islands was not been fully explored for actinomycetes diversity and bioactive compounds, hence the present study was planned to isolate the bioactive actinomycetes from samples collected from Elephanta, Radhanagar and Havelock beach of Andaman and Nicobar Islands and to screen for their antibacterial potential and bioactive secondary metabolites.

MATERIALS AND METHODS

(i) Sampling sites of marine soil

Marine soil samples were collected from pollution free environment at three different stations of Andaman and Nicobar Islands namely Elephanta, Radhanagar and Havelock beach. The collected soil samples were whitish dry in colour and sandy in texture. It was collected at the depth of 15 cm and transferred to the laboratory under aseptic conditions and stored at 4°C until further use.

(ii) Isolation of actinomycetes from marine soil samples

Actinomycetes were isolated using Actinomycetes isolation agar (AIA) medium. Soil samples (1 g each) were serially diluted up to 10^{-5} and 100µl from each dilution was spread over the surface of the AIA medium using the spread plate technique. The plates were incubated at 28°C for 7- 21 days. After incubation the actinomycetes were purified and sub-cultured on AIA agar plates by quadrant streaking technique and stored at 4°C for further use.

(iii) Preparation of antibiotic fermentation broth

Solid state fermentation was used for antibiotic production using the pigmented actinomycetes isolate. For the preparation of inoculum, the actinomycetes isolate AN1 was streaked on the Actinomycetes isolation agar (AIA) plates and incubated at 28°C for 7 days. A loop full of spores were scrapped from the plate and inoculated in 5ml of distilled water after sterilization. About 30g of rice was added into 250ml conical flask and 15ml of distilled water was added and sterilized. Then inoculum (5ml of distilled water + spores of the AN-1 isolate) was added to the conical flask containing sterile rice[8]. The flasks were incubated for 7-10 days (until the colour of pigment appears on the rice) at 28°C. After deep brown colour

formation on the rice, the fermented biomass was mixed with ethyl acetate and incubated for 1 day at 28°C on a rotary shaker at 110 rpm. Then the solvent is evaporated by rotary vacuum and the concentrated EA extract was collected and dried. The quantity of the extract was measured and stored in the sterile vials.

(iii) Bacterial strains

All pathogens used in this study were ATCC cultures which includes Gram positive organisms *Staphylococcus aureus* (ATCC 33591), *Bacillus cereus* (ATCC 14579) and Gram negative organisms *Salmonella paratyphi* A (ATCC 9150), *Klebsiella Pneumoniae* (ATCC 33495), *Shigella flexneri* (ATCC 12022), *Shigella boydii* (ATCC 9207), *Pseudomonas aeruginosa* (ATCC 27853) and *Proteus vulgaris* (ATCC 6380), *Escherichia coli* (ATCC 25922).

(iv) Antibacterial activity

The antibacterial activity was determined by well diffusion method. Pure culture of actinomycetes was inoculated in ISP 1 (International *Streptomyces* Project) broth and incubated at 28°C for 7 days on a rotary shaker at 110 rpm. Bacterial cultures were grown on nutrient agar medium. The culture (24h) of test organisms was swabbed on Muller Hinton agar (MHA). Wells were cut using sterile well borer on the agar surface and fermentation broth (200µl) was added to each well aseptically. Petri dishes were incubated for 24 hours at 30°C. The inhibition zones formed were visually detected and measured (mm) after 24 hours.

(v) Morphological, cultural and biochemical characterization

The active isolate was subjected to biochemical characterization to identify the species. Gram staining was performed to identify the morphological characterization of the isolate using cover slip method. Cultural characteristics of actinomycetes were tested by growing the isolate on 10 different mediums and the growth of the isolate, aerial and substrate mycelium colour, pigment production, reverse side pigment, diffusible pigments were recorded. Various biochemical tests were also performed to characterize the actinomycetes using citrate utilization, triple sugar iron, voges proskauer, melanin production, oxidase, urease, methyl red, indole production tests according to the International *Streptomyces* project[9]. The isolates were tested for its ability to utilize the carbon sources like arabinose, mannitol, dextrose, cello biose, inositol, lactose, salicin and raffinose.

RESULTS

(i) Antibacterial assay

Totally 8 actinomycetes were isolated from soil samples collected from three different sites of Andaman & Nicobar Islands. These isolates were subjected to antibacterial screening assay. Out of 8, one isolate AN1 exhibited broad spectrum antibacterial activity against standard pathogenic bacteria (Table 1).

Table 1
Antibacterial activity of Streptomyces sp. AN1 against standard bacterial pathogens

Bacterial pathogens	Zone of inhibition (mm)	
	Cell free supernatant	Ethyl acetate extract
Gram positive bacteria:		
Staphylococcus aureus (ATCC 33591)	19±1.52	22.16±1.04
Bacillus cereus (ATCC 14579)	17.16±1.04	19.5±0.5
Gram negative bacteria:		
Salmonella paratyphi A (ATCC 9150)	16.5±0.5	19.66±1.52
Klebsiella pneumoniae (ATCC 33495)	16.7±1.52	12.16±1.04
Pseudomonas aeruginosa (ATCC 27853)	13.1±1.04	12.83±0.28
Shigella flexneri (ATCC 12022)	13.1±1.04	12.2±0.28
Shigella boydii (ATCC 9207)	13.8±0.29	12.0±0.5
Proteus vulgaris (ATCC 6380)	12.7±1.15	9.83±0.29
Escherichia coli (ATCC 25922)	11.7±0.57	12.2±0.76

Values are mean ± S.D (n=3).

Cell free supernatant of AN1 isolate exhibited significant activity against *Staphylococcus aureus* (19mm), *Bacillus cereus* (17.16mm), *Salmonella paratyphi* A (16.5mm), *Klebsiella Pneumonia* (16.7mm) followed by *Pseudomonas aeruginosa* (13.1mm) *Shigella flexneri* (13.1mm), *Shigella boydii* (13.8mm), and *Proteus vulgaris* (12.7mm) and *Escherichia coli* (11.7mm). The EA extract showed more significant antibacterial activity than cell free extract against *Staphylococcus aureus* (22.6mm), *Bacillus cereus* (19.5mm), *Salmonella paratyphi* A (19.66mm) followed by *Klebsiella Pneumoniae* (12.16mm), *Pseudomonas aeruginosa* (12.83mm) *Shigella flexneri* (12.2mm), *Shigella boydii* (12mm), *Proteus vulgaris* (9.83mm) and *Escherichia coli* (12.2mm).

(ii) Morphological, cultural and biochemical characterization of AN1 isolate

The isolate AN1 colonies were large, pinkish white to red in colour, powdery with irregular margin in AIA medium. Out of different media tested for maximal growth AIA was found to be optimum (Fig 1A). The microscopic examination of the isolate AN1 under light microscope exhibited a hook like structure under 100X magnification and the spores are smooth, appeared as long chain and oblong in shape (Fig 1B).

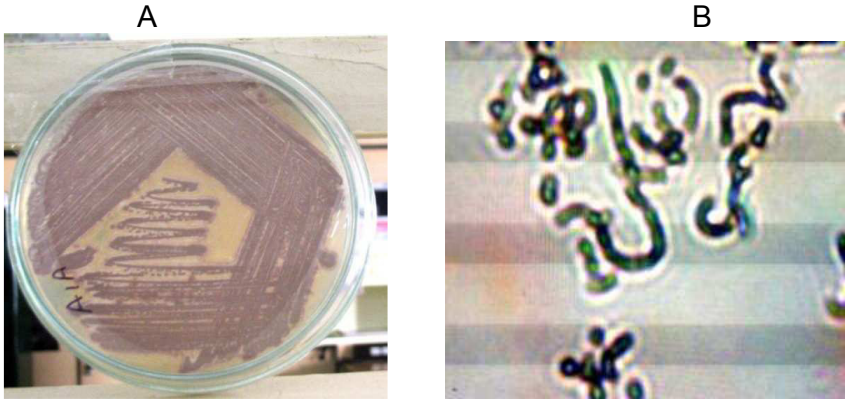

Figure 1
Streptomyces AN1 isolate A) AIA media B) morphology of the isolate under light microscope (100X).

The growth characteristics of AN 1 isolate on different ISP medium exhibited different colour aerial and substrate mycelium (Table 2).

Table 2
Cultural characteristics of Streptomyces isolate AN1 on different media

Medium	Growth pattern	Aerial mycelium
ISP 1	Good	Pink- red
ISP 2	Moderate	Pink
ISP 3	Abundant	Light brown- red
ISP4	Poor	White
ISP 5	Good	Light brown –pink
ISP 6	Poor	White
ISP 7	Good	Light brown- pink
AIA	Abundant	Light brown - red
SCA	Poor	White
Nutrient agar	Poor	White

The physiological and biochemical characteristics of the potent isolate are given in the Table 3. The isolate AN1 was Gram positive and produces pinkish white aerial mycelium and substrate mycelium and produced red colour pigment. The isolate reduced nitrate and positive for urease, oxidase and triple sugar iron. Growth of the isolate was found to be maximal when mannitol, cellobiose and inositol used as a carbon source. The optimum temperature was 28°C, pH was 7.0 and NaCl was 3%.

Table 3
Biochemical characteristics of the potential isolate AN1

Properties	AN1
Gram strain	+
Motility	-
Morphological characteristics	
Aerial mycelium	Pink white
Substrate mycelium	White
Colony colour	Pink red
Production of pigments	Red
Reverse side pigment	-
Spore chain	Hook like
Spore surface	Smooth
Biochemical characteristics	
Indole production	-
Methyl red	-
Citrate utilization	-
Nitrate reduction	+
Urease	+
Oxidase	+
Voges proskeur	-
Melanin production	-
Triple sugar iron	+
Carbon source utilization	
Arabinose	++
Mannitol	+++
Dextrose	++
Cellobiose	+++
Inositol	+++
Lactose	++
Salicin	++
Raffinose	++
Physiological characteristics	
Optimum Temperature for growth	28°C
Optimum pH	7
Growth in the presence of NaCl	
3%	++
5%	+
7%	+
9%	-
11%	-

+ Positive; – Negative; Good +++; fair ++

DISCUSSION

Natural product drug discovery using marine microbes prevailed for many years in pharmaceutical industry. Among the diverse marine microbial communities, actinobacteria have occupied a prominent and significant position as potential producers of structurally complex and unique metabolites. Most of the studies using actinomycetes were restricted to isolation, identification and maintenance of the isolate and only few reports are available on antagonistic activity of actinomycetes isolated from Andaman & Nicobar Islands. The antimicrobial activity exhibited by the AN1 isolate against tested bacterial pathogens was found to be more promising. Several reports have shown that *S. aureus* strains considered as a versatile pathogen known for causing nosocomial infections resulting in increased morbidity and mortality [10] and *B. cereus* is a virulent strain causing diseases in humans [11].

The extent of inhibition was greater in the case of ethyl acetate extract than cell free supernatant. The most pathogenic gram negative bacteria *S. aureus* and *B. cereus* were more susceptible to inhibition by EA extract (1mg/ml). Among the Gram positive test organisms *Salmonella paratyphi* A was more susceptible to inhibition by EA extract (1mg/ml). Solid state fermentation is an important tool for secondary metabolite extraction using actinomycetes[12]. The isolate AN 1 produced a dark reddish brown pigment in the rice medium. A similar activity by a pigment producing isolate *Streptomyces hygroscopicus* was reported[13]. S. *hygroscopicus* showed high bactericidal activity

against drug resisted pathogens such as MRSA, VRSA and ESBL strains. The isolate AN1 is identified as aerobic, Gram positive, produced a pinkish white aerial mycelium, smooth surface, spore forming and hook like structure and other cauterization including biochemical and physiological revealed that AN1 isolate belonged to the genus *Streptomyces* species. A number of studies have been reported that *Streptomyces* species are potent producer of medically important compounds. A moderately halophilic actinomycete strain isolated from marine sediments collected at the Marakkanam coast of Tamil Nadu exhibited moderate antibiosis against bacterial pathogens [14]. They have also reported that the Gram negative organisms are more susceptible to inhibition by the VITSDK1 EA extract than Gram positive organisms. Actinomycetes isolated from Western Ghats of South India have been shown to possess significant antibacterial activity against tested bacterial pathogens [15].

CONCLUSION

Based on result it can be concluded that the pigmented isolate AN1 isolated from the Andaman and Nicobar Island has broad spectrum of antibacterial activity. The isolate was designated as *Streptomyces* sp. VITAN1 based on the results of biochemical, cultural and morphological characterization. Further the isolate was found to be a promising source for producing antibacterial antibiotic which needs to be studied further. Molecular taxonomic identification of the isolate AN1, pigment extraction, purification, identification of chemical nature of the compound are under progress.

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