

ANTIMICROBIAL ACTIVITY OF *STREPTOMYCES VARIABILIS* STRAIN-VITUMVB03 ISOLATED FROM KANYAKUMARI MARINE SEDIMENTS

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ABSTRACT

Objective: This study was focused on an evaluation of antimicrobial activity of actinobacteria isolated from Kanyakumari marine sediments.

Methods: This study includes sample collection from Kanyakumari beach, followed by isolation of marine *Actinobacteria*, screening for antibacterial activity and antifungal activity. Finally, the most active isolate was identified using 16S rRNA sequencing.

Results: Marine *Actinobacteria* are one of the leading microbes of the ocean known for producing excellent secondary metabolites and having a broad spectrum of antimicrobial activity; hence, they are very important from an industrial point of view. The marine sediment sample used was collected from Kanyakumari beach, in the month of July, falling in the latitudinal range of 8.5074°N and in the longitude of 76.9730°E. A total of eight actinobacterial colonies were isolated by spread plate technique on *Actinomycetes* isolation agar, Kuster's agar, and starch casein agar. The isolate cultures were designated as UST1-UST8. Among them, UST3 showed a maximum zone of inhibition against clinical pathogens *Salmonella typhi* and *Aspergillus niger* with a zone of inhibition of 22 mm and 20 mm, respectively. In solvent extraction study, ethyl acetate and hexane was found as best solvents for extraction. Potential isolate UST3 was identified as *Streptomyces variabilis* using morphological studies and 16S rRNA sequencing. Potential strain was submitted as *S. variabilis*-VITUMVB03.

Conclusion: Results obtained in this study concluded that marine *Actinobacteria* have broad-spectrum antimicrobial activity and can be used in the pharmaceutical industry.

Keywords: Antifungal activity, Antibacterial activity, *Salmonella typhi*, *Aspergillus niger*, *Streptomyces variabilis*.

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INTRODUCTION

More than 75% of our earth surface is covered with water, of which major parts comes under marine environment. Marine environment is already proven as a rich source for the production of bioactive compounds [1]. Among the bioactive compound producing organisms present in marine environments, *Actinobacteria* were found as the most potent group microorganisms producing a large variety of bioactive compounds such as antifungal, antibacterial, antioxidant, and antiviral agents [2]. Marine *Actinobacteria* are the most inventive producers of bioactive compounds. These highly active *Actinobacteria* have applications not only medical field but also have shown their potential in various other fields such as agriculture and various industries [3]. *Actinobacteria* are spore producing Gram-positive bacteria with high G+C content in their DNA [4]. Marine *Actinobacteria*, when compare with terrestrial *Actinobacteria* are highly active, can produce different types of bioactive compounds and also have differences in their characteristics. These differences are due to the harsh marine environmental condition of marine *Actinobacteria* [5]. Most marine *Actinobacteria* of medical and economical applications comes under the subclass *Actinobacteridae*. In this subclass marine *Streptomyces* sp. are known as a source for the production of 60-70% of antibiotics. Most of the secondary metabolites that are used as antibacterial, antifungal, antiviral, antithrombotic, antitumor, and enzyme inhibitors are produced by *Streptomyces* sp. [6-8]. Furthermore, few available insecticides, herbicides, fungicides, and growth promoting substances for animals and plants are synthesized using marine *Streptomyces*.

After the discovery of penicillin and streptomycin antibiotics, many researchers felt the importance and need for the discovery of new drugs. Related to that importance various organisms were tested for

their ability to produce antimicrobial compounds. Among all tested microorganisms marine *Actinobacteria* stood out as potential producers of many antimicrobial and antitumor compounds [9,10]. Antibacterial compounds produced from chemical means are very efficient in controlling many infections and diseases, but these bacterial pathogens are soon getting resistant to these drugs [11]. Therefore, at present, synthesis of naturally or biologically synthesized bioactive compounds are needed. In this case, marine *Actinobacteria* are seen as the most promising source. Most of these organisms show a broad spectrum activity, for example, bonactin drug isolated from *Streptomyces* sp. possess very good antifungal and antibacterial activity [12]. Similarly, marinomycin drug produced from *Marinispora* sp. have shown good antifungal and anticancer activities [13]. In the present world, fungal disease is on raise when compared with bacterial disease since fungal disease are difficult to treat due to the similarity in the mammalian and fungal cell structures [14]. Marine *Actinobacteria* are also viewed for its ability as a potential antifungal agent. Commercially available antifungal drugs such as neomycin, griseofulvin, and polyene antimycotics are used as applicants and cannot be taken orally. Hence for the production of oral drug with least side effects for fungal disease marine *Actinobacteria* has been tested [15]. Various conducted tests proved that marine *Actinobacteria* can be used as a useful biological tool for the production of antifungal drugs against pathogenic fungi [16]. Recently, antifungal activity of highly biocompatible form chitinase and its derivatives that are used in biomedical fields such as wound healing, cartilage tissue engineering, drug delivery, and nerve generation are synthesized by marine *Actinobacteria* [17-19]. N-(2-hydroxyphenyl)-2-phenazinamine is a new and active antibiotic isolated from *Nocardia dassonvillei* which showed significant antifungal activity against *C. albicans* [19,20]. Hence, this study was focused on antibacterial and antifungal activities of marine *Actinobacteria* isolated Kanyakumari marine sediments.

METHODS

Chemicals and media

All the chemicals and media used in this study were purchased from HiMedia Laboratory Pvt. Ltd., Mumbai.

Sample collection

The marine sediments were collected from the Kanyakumari beach (8.5074°N, 76.9730°E), Tamil Nadu, India. Samples were collected from at a depth of 10-15 cm from the topmost layer and aseptically transferred into sterile bags. All samples were then transferred to microbiology laboratory and stored at 4°C for further studies.

Isolation of Actinobacteria

Isolation of marine *Actinobacteria* from the collected sample was done using *Actinomycetes* isolation agar, starch casein agar, and Kuster's agar by spread plate technique by using 50% of marine water. All plates were supplemented with a small amount of nalidixic acid and potassium dichromate to avoid both bacterial and fungal contamination, respectively. All plates were then incubated at room temperature for 5-7 days. Plates were checked for powdery and leathery colonies followed by isolation, purification and storage at 4°C for further use [21].

Clinical culture

Bacterial pathogens and fungal pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella typhi*, *Aspergillus flavus*, and *Aspergillus niger* were collected from Narayani Hospital, Sripuram, Vellore, Tamil Nadu.

Fermentation media

All eight isolates were separately inoculated in 100 ml production media (soluble starch, glucose, yeast extract, trace elements, and distilled water) in a 250 ml conical flask at 37°C in a shaker for 7 days at 120 rpm. After incubation period, all flasks were centrifuged at 1000 rpm for 15 minutes and supernatant was collected to carry of bioactive activities.

Antimicrobial activity

Antibacterial activity of actinobacterial isolates

Agar well diffusion method was used to determine the antibacterial activity of the crude extract. Using a sterile gel borer 6 mm diameter of the well was made on agar media. Inoculums of clinical pathogens (0.1 ml) were spread on the plates, and 100 µl of crude extract (20-200 µg/ml) was loaded in different wells to evaluate their activity against clinical pathogens such as *S. aureus*, *E. coli*, *S. typhi*, and *K. pneumoniae*. Plates were incubated for 24 hrs at 37°C. After 24 hrs, plates were checked for zone of inhibition [22,23].

Antifungal activity of actinobacterial isolates

Actinobacterial isolate UST-3 was inoculated in production media and incubated for 5 days at 28°C. After incubation, the cultures were centrifuged at 10,000 rpm for 15 minutes, and the supernatant was collected for extract production. Inoculums of fungal pathogens (0.1 ml) were spread on the plates, and 100 µl of crude extract (20-200 µg/ml) was loaded in different wells to evaluate the antifungal activity against *A. niger* and *A. flavus*. Plates were incubated for 24 hrs at 37°C. After 24 hrs, plates were checked for zone of inhibition [21].

Solvent extraction

Solvent extract of the bioactive compound was done using four different solvent such as butanol, ethyl acetate, hexane, and chloroform. The potential actinobacterial isolate UST-3 was inoculated in production media and incubated at 37°C for 7 days. After incubation period, the broth was centrifuged at 1000 rpm for 15 minutes and supernatant was collected. To the collected supernatant, equal volume of various solvent was added separately and kept in a rotary shaker for 24 hrs to extract the active compound. The extracted compound from each solvent was then tested for their activity against *S. typhi* and *A. niger* by

agar well diffusion method. After incubation, the zone of inhibition was measured [24].

Identification of potential marine actinobacterial strains

Identification of the potential isolates showing good antimicrobial and antioxidant activity was done using the key of Nonomura [25] and methods of Shirling and Gottlieb [26]. Along with this species level identification was also confirmed using cell wall composition analysis and by micromorphological studies [27,28]. Finally, the complete identification was done using molecular characterization based on 16S rRNA sequencing (Yaazh Xenomics, Chennai, Tamil Nadu, India). Bacterial genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation Kit. The fragments were amplified bi-directionally using the forward (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse (5'-TACGGYTACCTTGTACGACTT-3') primers (MJ Research Peltier Thermal Cycler). The sequence was analyzed by ABI 3730xl capillary DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan). Finally, the sequence was observed for similarity using NCBI BLAST similarity tool.

Statistical analysis

Using two-way analysis of variance values were analyzed between groups of same or different dpi. All values are shown as the mean±standard deviation. The significance of the differences between the mean values was determined using Student's t-test. For all statistical analysis, *p<0.05 were considered significant.

RESULTS AND DISCUSSION

Isolation of marine Actinobacteria

Isolation of marine *Actinobacteria* was done on actinobacterial isolation agar, starch casein agar, and Kuster's agar. The result obtained in this study reveals that the salt pan sediment samples are rich in actinobacterial population. From the collected samples, a total of eight actinobacterial colonies were isolated (Fig. 1). Considering the total number of isolates, the maximum number were isolated on actinobacterial isolation agar (4), followed by starch casein agar (2) and Kuster's agar (2). The isolates were identified using culture characteristics as mentioned in ISP manual. All eight isolates were belonging to the genera *Streptomyces* sp. In a previous study, eight actinobacterial colonies were isolated on *Actinomycetes* isolation agar from Andaman and Nicobar Islands sediment samples [29]. A total of 21 actinobacterial colonies were isolated on *Actinomycetes* isolation agar from the marine sediments isolated from Mahabalipuram sea shore [30]. In our study, also out of eight actinobacterial colonies four were isolated on *Actinomycetes* isolation agar followed by starch casein agar and Kuster's agar.



Fig. 1: Potential *Actinobacterial* isolated from Kanyakumari marine sediments

Antibacterial activity of actinobacterial extract

The bioactive potential of all eight isolates was evaluated by testing their antibacterial potential against Gram-positive and Gram-negative bacteria. Among eight only one isolate UST-3 showed antimicrobial activity against all test pathogens. Cell-free supernatant of UST-3 showed maximum inhibition against *S. typhi* with a zone of inhibition of 22 mm, followed by *K. pneumoniae* (16 mm) and *E. coli* (14 mm). The antibacterial profile shown by isolate UST-3 is shown in Fig. 2. The extract showed maximum activity at a dose of 200 µg/ml against all test pathogens. In a previous report marine *Streptomyces* sp. isolated from Royapuram, Muttukadu and Mahabalipuram sea shore showed activity < 13 mm against many clinical bacterial pathogens [30]. In another study marine *Streptomyces* sp. isolated from the southeast coast of India, Ramanathapuram, Sethu Karai, showed every good activity against clinical pathogens such as *S. aureus*, *S. typhi* and *E. coli* with a zone of inhibition above 20 mm. Maximum activity was shown against *S. typhi* with an inhibition zone of 27 mm [31]. Hence, our study is in accordance with the previous reports, and also UST-3 showed similar and better results when compared with the available reports.

Antifungal activity of actinobacterial extract

Isolate UST-3 showed good antifungal activity against *A. niger* and *A. flavus*. Cell-free supernatant of UST-3 showed a maximum zone of inhibition (20 mm) against *A. niger* at a dose of 100 µg/ml followed by 15 mm of inhibition at a dose of 80 µg/ml. Similarly, UST-3 showed maximum activity against *A. flavus* (14 mm) at a dose of 100 µg/ml followed by 80 µg/ml with a zone of inhibition of 6 mm (Fig. 3). In a previous study marine *Streptomyces* sp. isolated from marine sediments of Ennore, Chennai, Tamil Nadu, showed antifungal activity against *A. niger* and *A. flavus* with a zone of inhibition of 25 mm and 10 mm,

respectively [32]. Similarly, in another study antifungal activity of against *A. niger* with a zone of inhibition of 19 mm was reported using the crude extract of *Streptomyces variabilis* isolated from Thottada marine sediments, Kannur, Kerala [33]. In our study, also active isolate UST-3 showed the best activity against *A. niger*.

Screening using solvent extracted compound

In primary screening potential marine actinobacterial isolates UST-3 was selected for further studies, i.e., solvent extraction method. The isolate UST-3 showed a maximum zone of inhibition (27 mm) using ethyl acetate as solvent against the test organism *S. typhi* (Fig. 4). Similarly, UST-3 showed a maximum zone of inhibition (22 mm) using hexane as solvent against the test organism *A. niger* (Fig. 5). Here in this study, ethyl acetate and hexane was found as the best solvent for extraction of antibacterial and antifungal compounds from marine *Actinobacteria*. Ethyl acetate was reported as a suitable solvent for extraction of bioactive compounds from marine *Actinobacteria* [34], but in our study along with ethyl acetate, hexane also showed good active compound extraction ability.

Identification of potential isolate UST-3

Using 16S rRNA sequencing the potential isolate was identified as *S. variabilis*. The primary identification of potential strains done using the key of Nonomura, micromorphological studies and cell wall composition analysis also showed similar results (Table 1). Isolate UST-3 formed an extensively branched substrate mycelium which carried a recti flexible and smooth surface (Figs. 6 and 7). Potential isolate showed 99% of similarity with *S. variabilis* in the blast search analysis. The 16S rRNA sequencing of potential strains was confirmed that it occupies a distinctive phylogenetic position with the radiation, including representatives of the family using neighbor-joining tree. Potential strain was submitted in NCBI as *S. variabilis*-VITUMVB03.

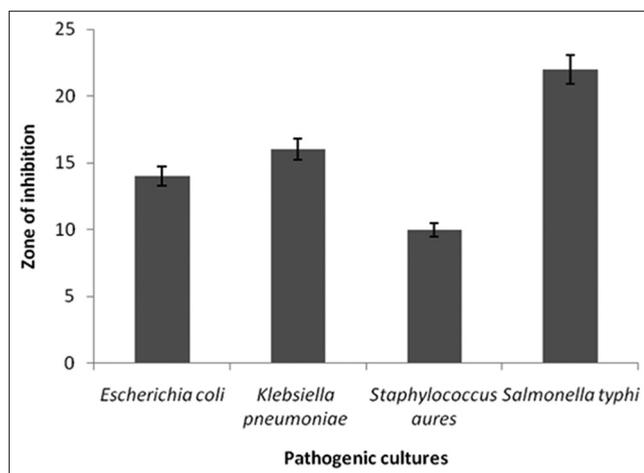


Fig. 2: Antibacterial activity of potential isolate UST-3 against clinical pathogens at 200 µg/ml dose

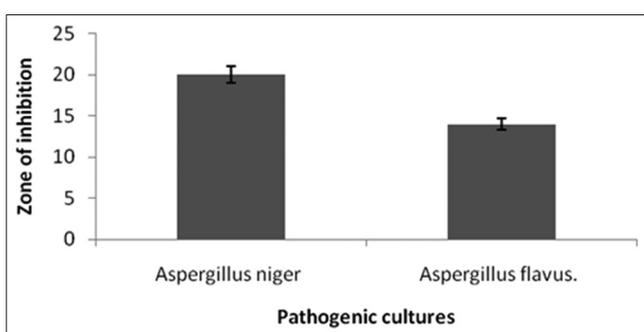


Fig. 3: Antifungal activity of potential isolate UST-3 against clinical pathogens at 100 µg/ml dose

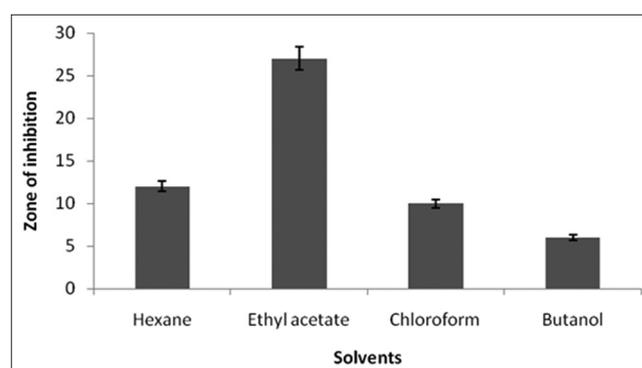


Fig. 4: Antibacterial activity of potential isolate UST-3 against *Salmonella typhi* using different solvents

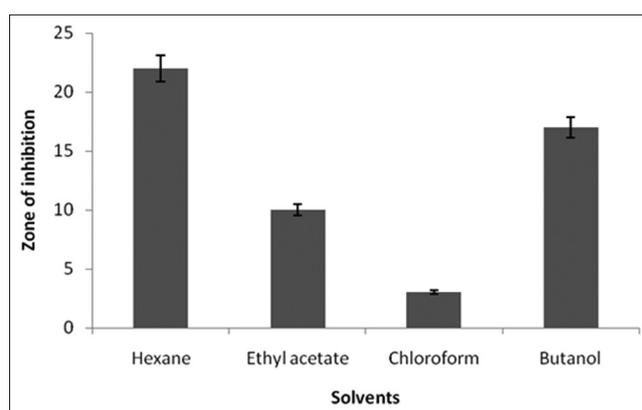


Fig. 5: Antifungal activity of potential isolate UST-3 against *Aspergillus niger* using different solvents

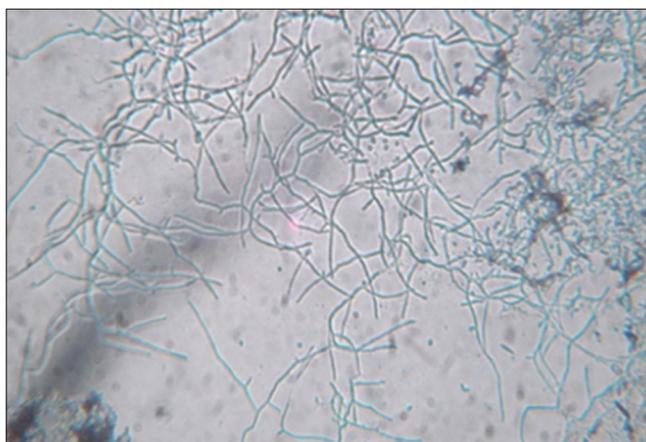


Fig. 6: Spore chain morphology of potential isolate UST-3

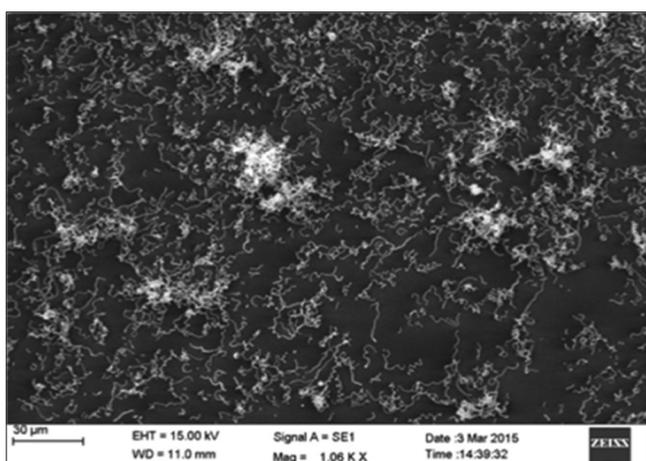


Fig. 7: Scanning electron microscopic image of potential isolate UST-3

Table 1: Morphological characteristic of UST-3

Characteristic	UST3
Gram staining	Off-grey
Color of aerial mycelium	-
Melanoid pigment	+
Reverse side pigment	-
Soluble pigment	-
Acid fast staining	-
Spore chain morphology	Recti flexible

CONCLUSION

Marine *Actinobacteria* are known producers of secondary metabolites; they are more miscellaneous and unique with this ability. The potential marine actinobacteria isolated in this study showed very good antibacterial and antifungal activities at a dose of 200 µg/ml and 100 µg/ml, respectively. Potential isolate *S. variabilis* isolated from Kanyakumari marine sediments can be used as a promising source for the development of antibacterial and antifungal drugs.

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