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ARTICLE

Antagonistic activity of terrestrial *Streptomyces* sp. VITNK9 against Gram negative bacterial pathogens affecting the fish and shellfish in aquaculture

Actividad antagonista de *Streptomyces* sp. VITNK9 contra patógenos bacterianos Gram negativos que afectan a los peces y mariscos en la acuicultura

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Resumen.- Se realizaron un total de 72 aislamientos de actinomicetos morfológicamente diferentes a partir de muestras recolectadas en diferentes regiones de Vellore, Tamil Nadu, India y seleccionados por su actividad antibacteriana contra patógenos de peces y mariscos. Todos los aislamientos de actinomicetos fueron examinados para determinar la actividad antibacteriana por el método de rayas cruzadas contra los patógenos seleccionados de peces y mariscos incluyendo *Aeromonas caviae, Aeromonas hydroplila, Edwardsiella tarda, Vibrio anguillarum*, *Vibrio harveyi*. El tamizaje secundario de aislamientos antagonistas por el método de difusión de pozos conduce a la identificación de aislamiento potencial. Las condiciones de cultivo para el aislamiento potencial fue optimizado para un crecimiento y rendimiento máximo del extracto bruto de acetato de etilo (EA). El aislamiento potencial se caracterizó por la taxonomía molecular y filogenia identificándose la especie como *Streptomyces* y nombrado *Streptomyces* sp. VITNK9. La secuencia de nucleótidos 16S rDNA fue buscada a través de la base de datos GenBank y mostró 83% de similitud con *Streptomyces vinaceusdrappus*. El extracto EA preparado a partir de *Streptomyces* sp. VITNK9 mostró actividad antagonista moderada a la que se accede por la zona de formación de inhibición del crecimiento, *Aeromonas caviae* (15,33 mm), *Aeromonas hydrophila* (17,66 mm), *Edwardsiella tarda* (18,33 mm), *Vibrio anguillarum* (14,33 mm) y *Vibrio harveyi* (14,33 mm). El valor de CIM del extracto de EA estaba comprendido entre 0,03 y 0,125 mg mL^{-1}. El espectro de GC-MS del extracto de acetato de etilo reveló la presencia de dos compuestos principales, pirrolo [1,2-a] pirazina-1,4-diona (56,67%) y hexahidro-3- (2-metilpropil) (27,91%), respectivamente. Los resultados del estudio sugieren que *Streptomyces* sp. VITNK9 es una fuente potencial de metabolitos secundarios antagónicos contra patógenos de peces y mariscos.

Palabras clave: *Streptomyces*, patógenos de peces y mariscos, actividad antibacteriana, metabolitos secundarios, análisis filogenético

Abstract.- A total of 72 morphologically different actinomycetes isolates were isolated from samples collected at different regions of Vellore, Tamil Nadu, India and screened for its antibacterial activity against fish and shellfish pathogens. All actinomycetes isolates were screened for antibacterial activity by cross streak method against the selected fish and shellfish bacterial pathogens including *Aeromonas caviae, Aeromonas hydroplila, Edwardsiella tarda, Vibrio anguillarum* and *Vibrio harveyi*. Secondary screening of antagonistic isolates by well diffusion method leads to the identification of potential isolate. Culture conditions for the potential isolate were optimized for maximal growth and yield of the ethyl acetate (EA) crude extract. The potential isolate was characterized by molecular taxonomy and phylogeny and identified as *Streptomyces* species and named as *Streptomyces* sp. VITNK9. The 16S rDNA nucleotide sequence was searched through the GenBank database and showed 83% similarity to *Streptomyces vinaceusdrappus*. The EA extract prepared from *Streptomyces* sp. VITNK9 showed moderate antagonistic activity accessed by the formation of zone of growth inhibition against, *Aeromonas caviae* (15.33 mm), *Aeromonas hydrophila* (17.66 mm), *Edwardsiella tarda* (18.33 mm), *Vibrio anguillarum* (14.33 mm) and *Vibrio harveyi* (14.33 mm). The MIC value of EA extract was ranged between 0.03-0.125 mg mL^{-1}. The GC-MS spectrum of the ethyl acetate extract revealed the presence of two major compounds, pyrrolo [1,2-A] pyrazine-1,4-Dione (56.67%) and Hexahydro-3-(2-Methylpropyl) (27.91%), respectively. The results of the study suggest that *Streptomyces* sp. VITNK9 is a potential source for antagonistic secondary metabolites against fish and shellfish bacterial pathogens.

Key words: *Streptomyces,* fish and shellfish pathogens, antibacterial activity, secondary metabolites, phylogenetic analysis

Introduction

Aquaculture appears to be one of the last frontiers by increasing its contributions to food security in the developing countries (Aly & Albutti 2014). Intensification of aquaculture has led to the conditions favouring the development of various fish diseases. In particular, fish bacterial diseases are responsible for heavy mortality in both wild and cultured fishes. This, in turn, leads to heavy financial losses to the fish farmers. Aquaculture management and disease control have become one of the major problems as the fish bacterial pathogens have become resistant to the conventional drugs being used in the aquaculture industry. Bacterial fish diseases such as hemorrhagic septicemia, edwardsiellosis, bacterial kidney disease, bacterial gill disease, pop eye, vibriosis, fin and tail rot were reported by different workers from different parts of the world (Selvakumar *et al.* 2010, Sihag & Sharma 2012). Vibriosis is a major disease caused by *Vibrio* sp. affecting all varieties of shrimps at all stages. The major species causing vibriosis in shrimp are *Vibrio alginolyticus, V. harveyi, V. anguillarum* and *V. parahemolyticus* (Goarant *et al.* 1999, Letchumanan *et al.* 2014, Wang *et al.* 2015, Santhakumari *et al.* 2016). Several strategies have been proposed in control of vibriosis. For example, vaccines have been developed, but generally, cannot be used as a universal disease control measure in aquaculture as they are too much time consuming and labour-intensive (You *et al.* 2005). So antibiotics and chemotherapeutics remain the method of choice for disease control in the aquaculture industry. Hence, there is an urgent need for the search of safe, effective and novel bioactive compounds from natural sources to deal with the fish bacterial pathogens and to control them in an eco-friendly manner (Thirumurugan & Vijayakumar 2013). Unexploited habitats still remain as a promising source for the discovery of novel bioactive compounds.

Microorganisms are playing a great role in the expansion of drug development. Especially, soil-dwelling microorganisms remain as an excellent resource for the isolation and identification of therapeutically important products. Among them, actinomycetes are extensively distributed in the soil providing many important secondary metabolites of high medical importance, commercial value, and diverse biological applications. Actinomycetes are aerobic, filamentous, spore-forming Gram-positive bacteria with high G+C (60-70%) content in DNA. They are the prime sources of novel bioactive compounds like antibiotics, enzymes and other bioactive compounds (Roshan *et al.* 2013). Extensive screening of terrestrial actinomycetes has also yielded many important drug leads. Actinomycetes produce more than half of the bioactive compounds in the antibiotic literature database (Lazzarini *et al.* 2000). The *Streptomyces* genera of the actinomycetes group are the dominant and primary antibiotic-producing organisms exploited by the pharmaceutical industry.

Streptomyces species are capable of forming heat and desiccation-resistant spores and also most of them are non-pathogenic to plants and animals. Hence, the *Streptomyces* species isolated from terrestrial origin has been considered as potential biocontrol agents (You *et al.* 2005). Approximately 7,600 bioactive compounds have been reported from *Streptomyces* species (Berdy 2005). Actinomycetes have also shown interesting activities in water such as degradation of starch and casein and production of antimicrobial agents. With such types of bioactivities, actinomycetes would play an important role in dealing with the fish bacterial pathogens (Zheng *et al.* 2000). There are studies on the bioactivity of actinomycetes, in particular, *Streptomyces* species against human and fish pathogens (Patil *et al.* 2001). Actinomycetes remain as one of the major natural source for novel and therapeutically bioactive compounds. Among them, many have been developed into drugs for the treatment of wide range of diseases of humans, plants and animals (Vaid & Sajeevan 2016). The objective of present investigation was the isolation, characterization and screening of actinomycetes isolated from the terrestrial regions of Vellore, Tamil Nadu, India, against fish and shellfish pathogens.

Materials and methods

Soil sampling

In the present study, soil samples were collected from different locations of Vellore district (12.9165°N, 79.1325°E), Ambur (12.9165°N, 79.1325°E), Madhanur (11.2777°N, 76.9920°E), Gudiyatham (12.9447°N, 78.8709°E) and Katpadi (12.9796°N, 79.1375°E), Tamil Nadu, India. The soil samples were collected at a depth of 5-10 cm under aseptic conditions in a sterile polyethylene bags and sealed tightly to avoid external contamination and transported immediately to the laboratory. The soil samples were air-dried and kept at 70 ºC for 30 min which stimulates the growth of actinomycetes by eliminating the vegetative Gram negative bacterial cells (Gebreyohannes *et al.* 2013, Janaki *et al.* 2014).

Isolation of actinomycetes

About 1 g of the pre-treated soil sample was serially diluted up to 10^{-6} dilution using conventional serial dilution technique. Aliquots containing 0.1 ml of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread and plated onto Actinomycetes Isolation Agar (AIA) (Sodium caseinate - 2.0 gL^{-1}, L-Asparagine - 0.1 gL^{-1}, Sodium propionate - 4.0 gL^{-1}, Dipotassium phosphate - 0.5 gL^{-1}, Magnesium sulphate - 0.1 gL^{-1}, Ferrous sulphate - 0.001 gL^{-1}, Agar - 15 gL^{-1}, adjusted to a final pH of 8.1 ± 0.2) and Starch Casein Agar (SCA) (Starch - 10 gL^{-1}, Casein powder 1 gL^{-1}, Sea water 37 gL^{-1}, Agar - 15 g L^{-1} adjusted to a final pH of 7.2 ± 0.2) (HiMedia Laboratories, Mumbai, India)[1] for the isolation of actinomycetes (Roshan *et al.* 2013). The plates were incubated at 28 ºC for 7-14 days. The actinomycetes colonies were selected based on the morphology and further sub-cultured (Thirumurugan & Vijayakumar 2013). The pure culture slants were maintained at 4 ºC in AIA until further use.

Test pathogens

The fish bacterial pathogens used in this study were *Aeromonas caviae* (MTCC 7725), *A. hydrophila* (MTCC 1739), *Edwardsiella tarda* (MTCC 2400) and *Vibrio harveyi* (MTCC 7954) which were obtained from Microbial Type Culture Collection and gene bank (MTCC), IMTECH, Chandigarh, India and *V. anguillarum* being a clinical isolate. The test pathogens *Aeromonas caviae*, *Edwardsiella tarda* and *A. hydrophila* were subcultured on nutrient agar medium (HiMedia, Mumbai, India)[1] whereas, *Vibrio harveyi* and *V. anguillarum* were subcultured on nutrient agar supplemented with 2% NaCl and incubated at 37 ºC for 24 h. The test pathogens were stored in nutrient broth containing glycerol stock at -20 ºC until further use.

Screening of antibacterial activity of actinomycetes against test pathogens

Primary screening

The antibacterial activities of the isolated actinomycetes were examined by cross streak method (Ganesan *et al.* 2016). The Muller Hinton Agar (MHA) plates were prepared. For determining the activity against *V. harveyi* and *V. anguillarum* the MHA plates were supplemented with 2% of NaCl. The actinomycetes were inoculated by a single streak in the center of the MHA plates and incubated at 28 ºC for 7 days. The test organisms were streaked right angles to the original actinomycetes isolate and the plates were incubated at 37 ºC for 24 h. Based on the zone of inhibition against the test pathogens, the potential actinomycetes were selected for secondary screening.

Secondary screening

The isolates which showed promising activity in the primary screening were inoculated into ISP 1 broth (Hi Media, India) and incubated in a rotary shaker (120 rpm) at 28 ºC for 7 days. The turbidity of the test bacterial suspension was adjusted to 0.5 McFarland standards in 0.85% saline. Lawn culture was spread using sterile swabs. Wells were bored using the wellbore on the MHA plates. The cell-free culture supernatant (CFS) (100 µL) obtained from the actinomycetes isolates was used to screen antagonistic activity against test pathogens by the well diffusion method. The culture plates were incubated at 37 ºC for 24 h and the zone of inhibition formed was measured in millimeters. All the experiments were repeated 3 times and the mean of the zone of inhibition was recorded. The actinomycetes isolate with potential antibacterial activity was selected for characterization and extraction of secondary metabolites.

Characterization of the potential isolate

Morphological characterization

Actinomycetes with potential antibacterial activity against the selected fish and shellfish pathogens were identified up to the genus level on the basis of their aerial mass color, reverse side pigments, melanoid pigments and spore chain morphology based upon Bergey's Manual of Determinative Bacteriology (Williams *et al.* 1989, Sharma 2014). The arrangement of spores in the mycelium was observed under 1000x magnification by cover slip method and the slides were observed under the light microscope (Thenmozhi & Kannabiran 2011). Gram's staining was also performed. The spore surface morphology was observed under scanning electron microscope.

[1]<http://www.himedialabs.com>

Culture characterization

The potential isolates were grown in different media such as Actinomycetes Isolation Agar, Glucose soyabean meal agar, ISP 1(Tryptone Yeast Extract Broth), ISP 2 (Yeast Malt Agar), ISP3 (Oat Meal Agar), ISP 4 (Inorganic Salt Starch Agar), ISP 5 (Glycerol Asparagine Agar Base), ISP 6 (Peptone Yeast Extract Iron Agar), ISP 7 (Tyrosine Agar), Kuster's agar, Muller Hinton agar, nutrient agar, Starch Casein Agar and Sabouraud's Dextrose Agar for optimization of media for maximal growth and bioactivity (Gebreyohannes *et al.* 2013).

Physiological characterization

The optimum pH, temperature and sodium chloride tolerance of the potential isolate were studied. The pH of the ISP 1 broth was adjusted to 3, 5, 7, 9 and 11. The flasks were inoculated with potential actinomycetes and incubated on a rotary shaker (120 rpm) for 7 days at 28 ºC. Four flasks (250 mL) containing ISP 1 broth were prepared and inoculated with actinomycetes and kept in the incubated rotary shaker (120 rpm) at different temperatures ranging between 25-60 ºC for a period of 7 days. The effect of sodium chloride on the growth of the isolate at various concentrations ranging between 0.5-13% was also analyzed.

Utilization of carbon sources

The carbon utilization ability of the potential isolate was studied using carbon utilization agar (ISP 9) supplemented with 1% of various carbon sources like arabinose, cellobiose, glucose, inositol, raffinose and salicin (Nonomura 1974). Carbon utilization agar is used for the characterization of *Streptomyces* sp. on the basis of carbon source utilized by the *Streptomyces* isolate.

Biochemical characterization

Various biochemical tests were performed including indole, citrate utilization, methyl red, Voges Proskauer's, melanin production, oxidase, catalase, gelatinase, urease, amylase, lipase tests for the identification of potential isolate (Abirami *et al.* 2013).

Molecular taxonomic characterization

The species level identification of the potential isolate was carried out by16S rRNA partial gene sequencing and construction of the phylogenetic tree. The genomic DNA of the isolate was extracted and 16S rRNA gene was amplified using a forward primer (27F-5'AGAGTTTGATCMTGGCTCAG3') and a reverse primer (1492R-3'TACGGYTACCTTGTTACGACTT5'). Sequencing reactions were performed using ABI PRISM® BigDyeTM terminator cycle sequencing kits. The obtained 16S rDNA nucleotide sequence of isolate VITNK9 was searched through the NCBI GenBank data base for sequence similarity using the blast search tool. The program MUSCLE 3.7 was used for multiple sequence alignment (Edgar 2004). The program Gblocks 0.91b was used to cure the resulting aligned sequences (Talavera & Castresana 2007). The phylogeny analysis was performed using the program Mega-6 software and the phylogenetic tree was constructed using the neighbor-joining method.

Secondary structure prediction and restriction site analysis of 16S rRNA gene

The secondary structure of the isolate VITNK9 was predicted using the Genebee online software and restriction sites in the 16S rRNA of the potential isolate were analyzed by NEB cutter Version 2.0 available online.

Large scale fermentation

The seed culture of the isolates which exhibited maximum activity in secondary screening was prepared by inoculating it into the ISP 1 broth, as the potential isolate grew excellently in ISP 1 medium (HiMedia, India)[1] and then incubating it under shaking condition at 28 ºC for 3 days (Abirami *et al.* 2015). After incubation, about 10 mL of the cultures were transferred into 1L of ISP 1 broth and incubated in an orbital shaker at 28 °C for 7 days.

Preparation of solvents extracts

The fermented media was filtered using Whatman no.1 filter paper and the cell-free culture supernatant (CFS) was collected in a sterile conical flask. Liquid -liquid extraction technique was used to obtain the crude extract from the cell-free culture supernatant. Various solvents, ethyl acetate, chloroform and petroleum ether was used for the preparation of extracts and each extract was concentrated using a rotary evaporator.

Antibacterial activity

The solvent extracts of the potential isolate VITNK9 were used to determine the antibacterial activity against the selected fish bacterial pathogens by the well diffusion method. The bacterial suspension of the test bacteria was adjusted to 10^6CFU mL^{-1} and a lawn culture was made on

Muller-Hinton agar plates. The crude extract of the isolate was diluted to a concentration of 100µg/100µL in dimethyl sulfoxide. Wells were bored using sterile wellbore on the agar plates and 100 µL of the solvent extracts were added to each well. Ciprofloxacin was used as positive control and respective solvents were also used as negative control. The plates were incubated at 37 ºC for 18-24 h. The zone of inhibition was measured in diameters to assess the antibacterial activity. All the experiments were performed in triplicates.

Determination of minimal inhibitory concentration (MIC)

The MIC of the ethyl acetate extract of the active isolate was determined by serial dilution method in a sterilized 96 well plate. The sterile Muller-Hinton Broth (100 µL) and 100 µL of the overnight culture of the fish bacterial pathogens were added to each well. The ethyl acetate extract was serially diluted from 1 mg mL^{-1} to 0.01 mg mL^{-1} for determining the MIC of the active isolate. The MIC was considered as the lowest concentration of which showed no increase in the optimal density (OD 570) read at the microplate reader (Bio-Rad model 680 Microplate reader)[2].

Gas chromatography and mass spectrometry of the ethyl acetate crude extract

The antibacterial bioactive compounds present in the ethyl acetate crude extract was identified by subjecting to the crude to GC-MS analysis using Perkin Elmer workstation Clarus 600GC coupled to a mass spectrometer Elite- 5MS (30m x 0.25mm) width film depth of 250 µm capillary tube was used. The instrument had an oven initial temperature of 55 ºC for 3 min and a ramp program which elevates from 6 ºC/min up to 310 ºC further 3 min of the isothermal hold. Helium was used as a carrier gas with a flow rate split ratio of 10:1. About 2 µL of sample was injected and the injector temperature maintained at 250 ºC. Total run time was 32 min. The mass spectrum obtained was compared with spectra available in NIST-LIB 0.5 (National Institute of Standards and Technology)[3] library for matching using the in-built software of GC-MS system (Wiley GC-MS-2007). The concentration of individual compounds present was expressed as percentages through peak area presentation.

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[3]<https://www.nist.gov/>

Results

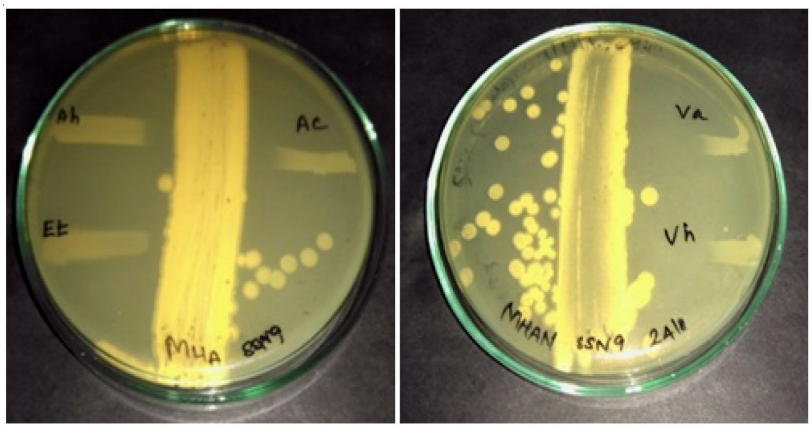
Isolation and screening of actinomycetes

Based on the colony morphology, a total of 72 actinomycetes were isolated from different terrestrial samples. Distinct actinomycetes colonies were observed in culture plates at 10^{-3} and 10^{-4} dilutions. Actinomycetes isolate exhibited different colony morphology and characteristics (size, shape, and colour) and the colonies were small to medium sized. The colour of the mature sporulating aerial mycelium ranged from white, gray, blue, light orange to violet. Brown, black and yellow coloured substrate mycelium was also recorded (Table 1). The isolate NN25 produced melanoid pigments. AIA or SCA agar media was used for isolation of all the 72 actinomycetes isolates. All the isolates were screened for antibacterial activity against the selected fish bacterial pathogens. About 18% of the isolates showed antibacterial activity in the primary cross streak method. Among them, only 7% of the isolates had abroad range of antibacterial activity against all the selected fish bacterial pathogens. The isolate VITNK9 exhibited abroad spectrum of activity against all the tested fish bacterial pathogens (Fig. 1). Those isolates with a broad range of antibacterial activity in primary screening were subjected to secondary screening by well diffusion method. Among all the isolates, the isolate VITNK9 showed a broad spectrum of activity against all the tested bacterial

Table 1. Cultural characteristics of potential actinomycetes isolates / Características de posibles aislamientos de actinomicetos en cultivo

S.No	Actinobacterial isolates code	Media for isolation	Color of aerial mycelium	Reverse side pigment
1	NN1	SCA	White	Purple
2	KM1	SCA	White	-
3	MN2	SCA	White	-
4	BN2	AIA	Greenish grey	Brown
5	BN3	SCA	White	-
6	BN4	AIA	Whitish grey	-
7	NK5	SCA	White	Pale yellow
8	NK6	AIA	Whitish grey	-
9	NK7	AIA	Grey	Black
10	VITNK9	AIA	Whitish grey	Pale yellow
11	NK11	SCA	Grey	Black
12	NK24	AIA	Whitish grey	-
13	NN25	AIA	White	Yellow

Figure 1. Primary screening of the isolate VITNK9 against the fish bacterial pathogens by cross streak method / Cribado primario del aislado VITNK9 contra los patógenos bacterianos de los peces por el método de raya cruzada



pathogens. The cell-free supernatant (CFS) of the isolate VITNK9 exhibited significant activity against *Aeromonas caviae* (16 mm), *Aeromonas hydrophila* (16.5 mm), *Edwardsiella tarda* (17 mm), *Vibrio anguillarum* (15 mm) and *Vibrio harveyi* (14 mm). The fish bacterial pathogen *E. tarda* was found to be more susceptible and *V. harveyi* was least susceptible for CFS treatment. The potential isolate was selected for further characterization.

Morphological characterization

The colonies of the isolate VITNK9 were medium to large sized, powdery with irregular margin on AIA culture medium (Fig. 2a). The colour of the aerial mycelium was white to gray and substrate mycelium was pale yellow. The complete growth of the isolate was observed on the 14th day. The isolate was Gram-positive and long chains of spores (oblong in shape) were observed under the light microscopic examination (1000X magnification). Smooth spore surface and hyphae with spiral spore chain morphology were observed under scanning electron microscope (Fig.2b). The isolate exhibited the typical structural features and morphology of *Streptomyces* sp. The morphological and biochemical characteristics of the isolate are given in Table 2.

Biochemical characterization

The growth of the isolate VITNK9 on various media is given in Table 3. The isolate was characterized by the methods recommended by International *Streptomyces* Project. The isolate grew in abundance on ISP 3 and ISP 4 agar media. The isolate also grew excellent in AIA, ISP 1,

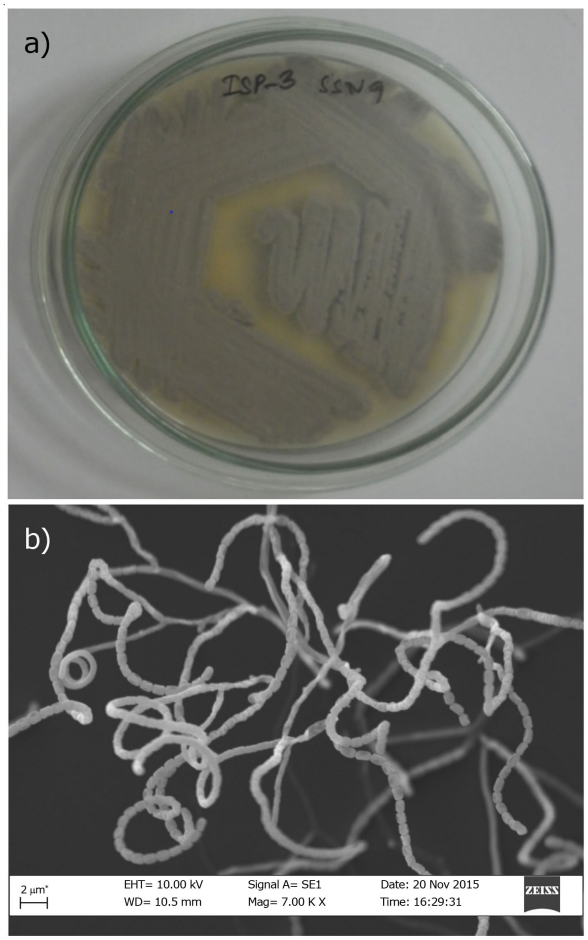


Figure 2. *Streptomyces* sp. VITNK9. a) Colony morphology and b) SEM spore morphology / *Streptomyces* sp. VITNK9. a) Morfología de las colonias y b) Morfología de las esporas de SEM

Table 2. Characterization of the potential isolate *Streptomyces* sp. VITNK9 / Caracterización del aislamiento potencial *Streptomyces* sp. VITNK9

Properties	
Presence of aerial and substrate mycelium	+
Sporophore morphology	Spiral
Spore surface	Smooth
Color of aerial mycelium	Grey
Color of substrate mycelium	Light brown
Shape of spores	Oblong
Melanin pigment	–
Indole production	–
Methyl red test	+
Vogesproskauer	–
Citrate utilization	+
Gelatin hydrolysis	–
Catalase	+
Oxidase	+
Urease	+
Starch hydrolysis	+
H_2S production	–
Nitrate reduction	–
TSI	k/k
Carbon source utilization	
Arabinose	+++
Cellubiose	+
Glucose	+++
Inositol	+++
Mannitol	+++
Raffinose	+
Salicin	+++

+ Positive , - negative, k/k alkaline slant, alkaline butt; +++ good; + Fair

glucose soya meal broth, Kuster's agar and Nutrient agar. Good to moderate growth of the isolate was observed in SCA, ISP 5, ISP 6 and ISP 7. Poor growth was observed in ISP 2 and SDA media.

Different biochemical tests were carried out to identify the isolate. The isolate was found to be positive for methyl red test and citrate utilization test and negative for indole, Voges Proskauer's test and triple sugar iron test. It produced amylase, catalase, oxidase and urease, and was negative for nitrate reduction and gelatinase and lipase production. It utilized arabinose, glucose, inositol, mannitol, and salicin as carbon sources for maximal growth and cellubiose and raffinose were not used by the isolate. The culturing conditions were optimized with respect to culture media, pH, temperature and NaCl concentration. The morphological and cultural characterization of the potential isolate revealed the nature and genera of the isolate.

Table 3. Characteristics of the isolate *Streptomyces* sp. VITNK9 on different media / Características del aislamiento de *Streptomyces* sp. VITNK9 en diferentes medios de cultivo

Media	Growth	Aerial mycelium	Substrate mycelium
ISP-1	Excellent	Creamy white	-
ISP-2	Poor	Creamy white	White
ISP-3	Abundant	Powdery grey	Grey
ISP-4	Abundant	Grey	Grey
ISP-5	Moderate	Grey	Grey
ISP-6	Good	Grey	White
ISP-7	Good	Grey	White
Nutrient Agar	Excellent	Whitish grey	White
SDA	Poor	Pale white	Colourless
SCA	Good	Powdery White	White
AIA	Excellent	White to grey colonies	Pale white
Kuster's agar	Excellent	Powdery white	Pale white
Glucose soya meal broth	Excellent	Creamy white	-

Molecular taxonomic characterization

The blast search of the 16S rDNA nucleotide sequence (876 bp) of the isolate with the NCBI, GenBank database revealed 83% similarity with *Streptomyces vinaceusdrappus* (KF554235). The 16S rDNA nucleotide sequence of the isolate was deposited in GenBank (NCBI) with an accession number (KX894540). Based on the molecular characterization and phylogeny of the isolate, it was confirmed to belong to the genus *Streptomyces* and designated as *Streptomyces* sp. VITNK9. The phylogenetic tree of the *Streptomyces* sp.VITNK9 is shown in the Figure 3.

Secondary structure prediction and restriction site analysis of 16S rRNA gene

The secondary structure for the isolate *Streptomyces* sp. VITNK9 was predicted by the greedy method. It was observed that the free energy of the thermodynamic structure was -194.8 kcal mol^{-1}, energy threshold was -4.0 with cluster factor 2, conserved factor 2, compensated factor 4, and conservativity 0.8. The isolate had 59 restriction enzyme sites.

Antibacterial activity of the solvent extracts

The isolate VITNK9 was extracted with different solvents including ethyl acetate, petroleum ether, and chloroform. The EA extract showed maximum antibacterial activity

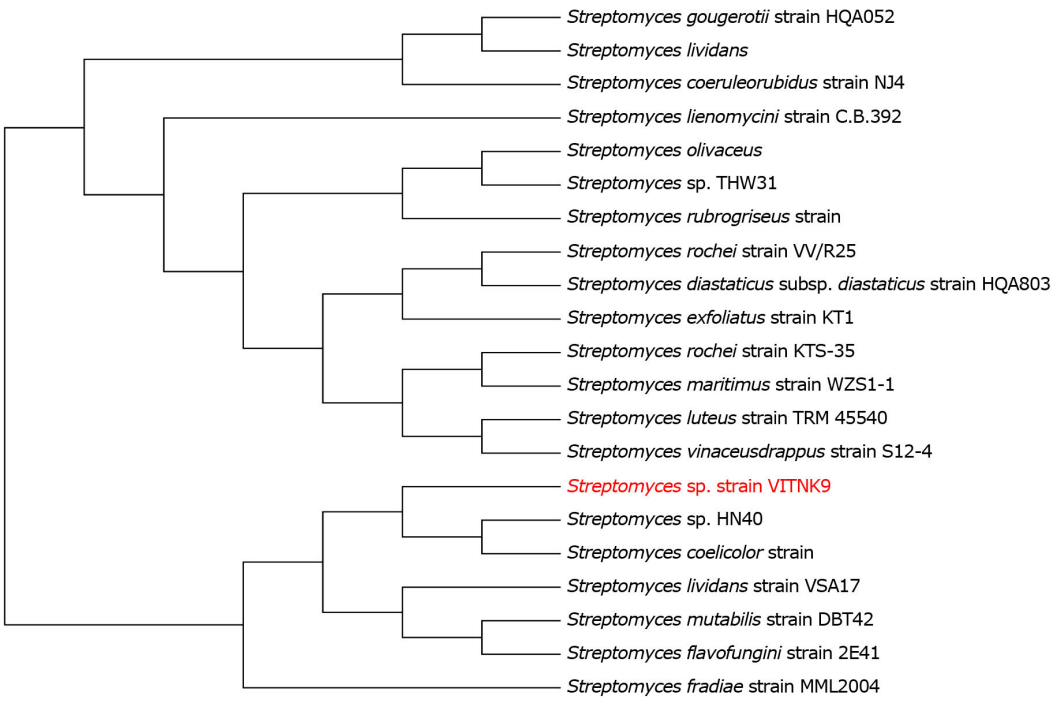


Figure 3. Phylogenetic analysis of *Streptomyces* sp. VITNK9 / Análisis filogenético de *Streptomyces* sp. VITNK9

when compared to other solvent extracts. The EA extract showed antibacterial activity against *Aeromonas caviae* (15.33 mm), *Aeromonas hydrophila* (17.66 mm), *Edwardsiella tarda* (18.33 mm), *Vibrio anguillarum* (14.33 mm) and *Vibrio harveyi* (14.33 mm). The antibacterial activity observed with EA extract was comparatively higher than the activity observed with CFS (Table 4).

Determination of minimal inhibitory concentration (MIC)

The MIC value of the ethyl acetate extract prepared from the active isolate was ranged between 0.03 - 0.125 mg mL^{-1}. The MIC value of *A. hydrophila* and *E. tarda* was found to be 0.03 mg mL^{-1}, for *V. anguillarum* and *V. harveyi* was 0.06 mg mL^{-1} and for *A. caviae* was 0.125 mg mL^{-1}.

Gas Chromatography and Mass Spectrometry of the ethyl acetate crude extract

The partial characterization of the EA crude extract of VITNK9 was carried out by GC-MS and the chromatogram obtained is shown in the Figure 4. The GC-MS spectrum of the EA crude extract revealed the presence of two major

Table 4. Antibacterial activity of the actinomycetes isolate VITNK9 against fish pathogens by well diffusion method / Actividad antibacteriana del aislamiento de actinomicetos VITNK9 frente a patógenos de peces por método de difusión de pozos

Fish bacterial pathogens	Zone of inhibition (mm)		
	VITNK9 Cell free supernatant	EA extract	MIC of EA extract (mg mL^{-1})
A. caviae	14.57 ± 0.48	15.33 ± 0.60	0.13
A. hydrophila	16.28 ± 1.37	17.66 ± 1.30	0.03
E. tarda	17.00 ± 1.29	18.33 ± 1.70	0.03
V. anguillarum	14.71 ± 0.68	14.33 ± 0.66	0.06
V. harveyi	13.85 ± 0.63	14.33 ± 0.88	0.06

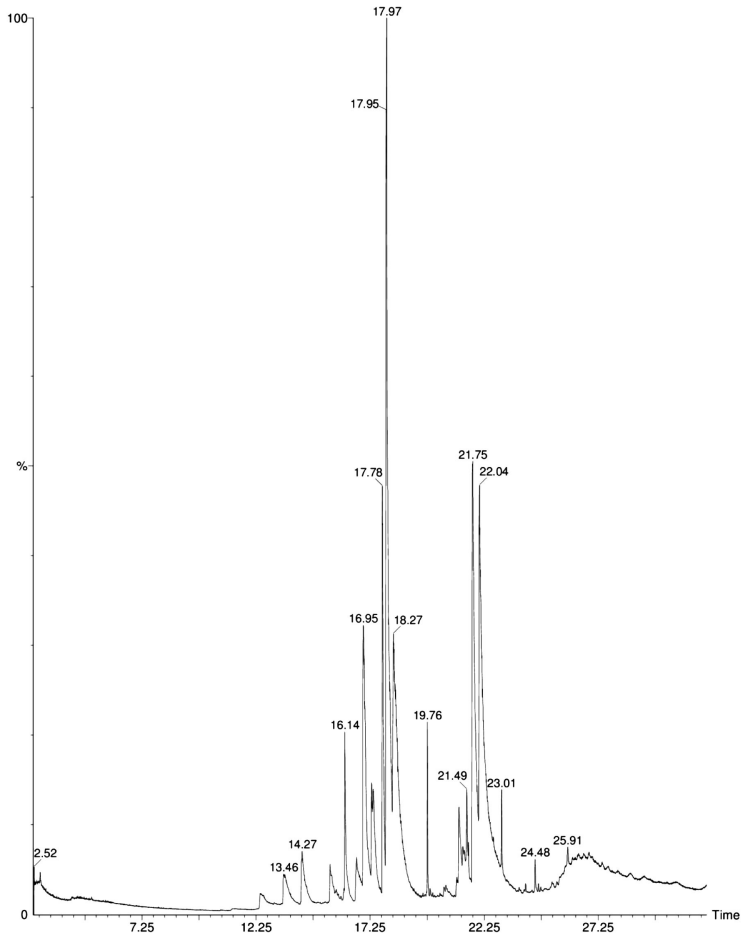


Figure 4. GC-MS chromatogram of VITNK9 ethyl acetate crude extract / Cromatograma GC-MS de extracto crudo de acetato de etilo de VITNK9

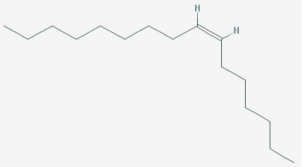
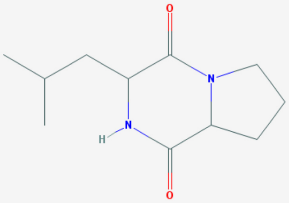
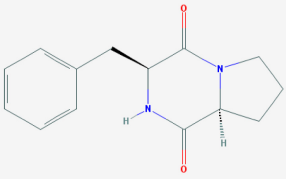
compounds (Table 5), pyrrolo[1,2-A] pyrazine-1,4-Dione hexahydro-3-(2-Methylpropyl) (56.67%) and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)-(27.91%) respectively. The four peaks at the retention time 16.95, 17.95, 17.97 and 18.27 min represents isomers of pyrrolo[1,2-A] pyrazine-1,4-Dione hexahydro-3-(2-Methylpropyl (NIST library matching). The peaks at the retention time 21.49 and 21.75 min represent the isomers of pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)- (NIST library matching).

Discussion

Actinomycetes are one of the most indispensable and vibrant bioactive compounds producing microorganisms found in both terrestrial and marine environments. These actinomycetes are fertile producers of antibiotics and new chemical entities as intracellular/ extra cellular secondary metabolites (Hassan *et al.* 2017). Soil samples have been screened for decades and only very few taxa of actinomycetes have been isolated and studied (Abd-Elnaby *et al.* 2016). The emerging bacterial diseases have hampered the growth of aquaculture tremendously. Hence, there is a need for developing antibacterial agents from natural sources to control and management of fish and shellfish bacterial diseases.

In the present study, 72 morphologically distinct actinomycetes were isolated from the terrestrial soil samples collected from Vellore, India. Among them, the potential isolate VITNK9 exhibited a significant antibacterial activity against fish and shellfish bacterial pathogens. Isolation of actinomycetes from the terrestrial environment has been inevitably reported earlier (Vaijayanthi *et al.* 2012, Cholarajan & Vijayakumar 2013, Vijayakumar & Malathi 2014). The isolate VITNK9 produced whitish gray aerial mycelium and pale yellow substrate mycelium. It also had an earthy odour which is the known characteristics of the *Streptomyces* sp. The colonies of actinomycetes with white and greyish aerial

Table 5. GC-MS analysis of the ethyl acetate extract of VITNK9 / Análisis GC-MS del extracto de acetato de etilo de VITNK9

Retention Time	Name of the compound	Molecular formula	MW	Area (%)	Suggested chemical formula
14.273	Octadecene (E)-	$C_{18}H_{36}$	252	2.590	
15.494	7-Hexadecene, (Z)-	$C_{16}H_{32}$	224	1.137	
16.139	5-Eicosene, (E)-	$C_{20}H_{40}$	280	2.694	
16.954 17.79 17.975 18.28	Pyrrolo[1,2-A]Pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}O_2N_2$	210	56.67	
21.49 21.746	Pyrrolo[1,2-A]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}O_2N_2$	244	14.120	

mycelium and yellow substrate mycelium have been reported earlier (Gebreyohannes *et al.* 2013). The isolate also grew excellently well on ISP3, ISP4, nutrient agar, Kuster's agar and AIA. The potential actinomycetes isolate VITNK9 was characterized by morphological, biochemical, physiological and molecular characterization. The isolate was identified based on the comparison with the keys for classification and identification by Nonomura and Bergey's manual of Determinative Bacteriology (Nonomura 1974, Williams *et al.* 1989). The 16S rRNA gene sequencing remains as a significant and reliable tool for the identification and confirmation of the actinomycetes up to the genus and species levels. The isolate was identified as *Streptomyces* sp. and designated as *Streptomyces* sp. VITNK9 on the basis of the molecular taxonomic characterization (Thirumurugan & Vijayakumar 2015, Augustine *et al.* 2016, Jenifer *et al.* 2018).

In the present study, 18% of the isolates showed antibacterial activity against the fish and shellfish bacterial pathogens. There are many reports which illustrate the antimicrobial activity of the actinomycetes isolated from different habitats (Cho *et al.* 2012, Chaudhary *et al.* 2013, Gebreyohannes *et al.* 2013, Ganesan *et al.* 2017). Antibacterial activity of actinomycetes against human as well as fish bacterial pathogens has been reported (Babuselvam *et al.* 2016, Jenifer *et al.* 2018). Mohan *et al.*(2016) have reported on the antimicrobial activity of actinomycetes extracts against the fish bacterial pathogens. Antibacterial activity of actinobacterial isolates against *A. hydrophila, A. sorbia* and *E. tarda* has already been reported (Patil *et al.* 2001).

In our study, among the various solvents used the ethyl acetate crude extract of the potential *Streptomyces* sp. VITNK9 had a broad spectrum antibacterial activity against the tested fish and shell fish bacterial pathogens. In a study, the protein extracted from *Streptomyces* spp. KV3 showed an inhibitory activity against *Vibrio harveyi* (17 mm) *Aeromonas hydrophila* (10 mm) and *Vibrio alginaticus* (9 mm) (Rani & Doss 2016). Similar findings were reported where the ethyl acetate extract of

actinomycetes showed antibacterial activity against fish bacterial pathogens (Patil *et al.* 2016a,b). The ethyl acetate crude extract of the potential isolate VITNK9 showed a broad range of antibacterial activity with the MIC value ranging between 0.03- 0.125 mg mL^{-1} against the fish and shellfish pathogens *A. caviae, A. hydrophila, E. tarda, V. harveyi* and *V. anguillarum*. The effectiveness of the antibacterial compounds does vary from one species to other, depending upon the factors like sampling site, media composition to the genetic potential of an organism. Tuan *et al.* (2017) have also reported the antibacterial activity shown by the endophytic actinomycetes (MTR 711, MTR 622 and MTL 121) with the MIC of 93.30 to 300 mg mL^{-1} against *A. hydrophila, A. caviae* and *S. agalactiae.* It was also reported that the partially purified bioactive compound (protein) showed moderate antibacterial activities against the fish bacterial pathogens *A. hydrophila, V. harveyi* and *V. alginolyticus* (Pushpa-Rani & Doss 2016).

The EA crude extract was subjected to GC-MS analysis to obtain a rudimentary idea of the secondary metabolites present in the extract. The GC-MS analysis revealed the presence of pyrrolo[1,2-A] pyrazine-1,4-dione, hexahydro-3-(2-Methylpropyl) in the EA crude extract which could be responsible for inhibiting fish bacterial pathogens. Antioxidant (free radical scavenging) activity of pyrrolo [1,2-A] pyrazine-1,4-dione has recently been reported (Ser *et al.* 2015). A report illustrates that *Streptomyces* sp. could produce actinonin with anti-VaPDF activity which provides resistance against *V. anguillarum* (Yang & Sun 2016). Antibacterial activity of EA extract containing Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- 3-(2-methylpropyl) extracted from *Streptomyces* sp. VITMK1 was recently reported from the authors lab (Manimaran *et al*. 2017). In the present study the peak area of pyrrolo [1,2-A] pyrazine-1,4-dione, hexahydro-3-(2-Methylpropyl) was found to be 56.67%. The pyrrolo compound was predicted and the structure was obtained with a molecular formula of $C_{11}H_{18}O_2N_2$ and a molecular weight of 210 kDa.

Streptomyces sp. VITNK9 isolated from the terrestrial soil sample found to be a rich source of antibacterial compounds against the fish and shellfish pathogens. The secondary metabolites (Pyrrolo [1,2-A] pyrazine-1,4-dione and hexahydro-3-(2-Methylpropyl) present in the ethyl acetate crude extract needs to be explored further to develop as an antibacterial agent against fish and shellfish pathogens.

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Literature cited

Abd-Elnaby H, G Abo-Elala, U Abdel-Raouf, A Abd-Elwahab & M Hamed. 2016. Antibacterial and anticancer activity of marine *Streptomyces parvus*: Optimization and application. Biotechnology and Biotechnological Equipment 30: 180-191.

Abirami M, VG Khanna & K Kannabiran. 2013. Antibacterial activity of marine *Streptomyces* sp. isolated from Andaman & Nicobar Islands, India. International Journal of Pharma and Bio Sciences 4: 280-286.

Abirami M, JV Gopal & K Kannabiran. 2015. Extraction and identification of antibacterial compound from marine *Streptomyces* sp. VITAK1 isolated from the coast of Andaman and Nicobar Islands, India. Applied Biochemistry and Microbiology 51: 406-410.

Aly SM & A Albutti. 2014. Antimicrobials use in aquaculture and their public health impact. Journal of Aquaculture Research and Development 5: 247.

Augustine D, JC Jacob & R Philip. 2016. Exclusion of *Vibrio* spp. by an antagonistic marine actinomycete *Streptomyces rubrolavendulae* M56. Aquaculture Research 47: 2951-2960.

Babuselvam M, A Panneerselvam, K Kanimozhi & G Kavitha. 2016. Antibacterial potential of actinomycetes from Seagrass against human and aquaculture pathogens. Journal of Microbiology and Biotechnology Research 6: 32-38.

Berdy J. 2005. Bioactive microbial metabolites. Journal of Antibiotics 58: 1-26.

Chaudhary HS, J Yadav, AR Shrivastava, S Singh, AK Singh & N Gopalan. 2013. Antibacterial activity of actinomycetes isolated from different soil samples of Sheopur (A city of central India). Journal of Advanced Pharmaceutical Technology & Research 4: 118-123.

Cho SS, YH Choi, JR Simkhada, P Mander, DJ Park & JC Yoo. 2012. A newly isolated *Streptomyces* sp. CS392 producing three antimicrobial compounds. Bioprocess and Biosystems Engineering 35: 247-254.

Cholarajan A & R Vijayakumar. 2013. Isolation, identification, characterization, and screening of antibiotic producing actino-bacteria from the crop field of Thanjavur district, Tamilnadu, India. International Journal of Science and Research 4: 55-60.

Edgar R. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32: 1792-1797.

Ganesan P, AD Reegan, RHA David, MR Gandhi, MG Paulraj, NA Al-Dhabi & S Ignacimuthu. 2016. Antimicrobial activity of some actinomycetes from Western Ghats of Tamil Nadu, India. Alexandria Journal of Medicine 53: 101-110.

Gebreyohannes G, M Feleke, S Sahile & N Raja. 2013. Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia.Asian Pacific Journal of Tropical Biomedicine 3(6): 426-435.

Goarant C, F Merien, F Berthe, I Mermoud & P Perolat. 1999.Arbitrarily primed PCR to type *Vibrio* spp. pathogenic for shrimp. Applied and Environmental Microbiology 65: 1145-1151.

Hassan SS, K Anjuma, SQ Abbas, A Akhter, BI Shaguftac, SAA Shaha & U Tasneem. 2017. Emerging biopharmaceuticals from marine actinobacteria. Environmental Toxicology and Pharmacology 49: 34-47.

Janaki T, BK Nayak & T Ganesan. 2014. Different pre-treatment methods in selective isolation of actinomycetes from mangrove sediments of Ariyankuppam back water estuary, Puducherry. International Journal of Advanced Research in Biological Sciences 1: 154-163.

Jenifer JA, R Remya, S Velmurugan, M Michaelbabu & T Citarasu. 2018. *Streptomyces castaneoglobisporus* AJ9, A haloalkaliphilic actinomycetes isolated from solar salt works in southern India and its pharmacological properties. Indian Journal of Geo-Marine Sciences 47: 475-488.

Lazzarini A, G Cavaletti & F Marinelli. 2000. Rare genera of actinomycetes as potential producers of new antibiotics. Antonie Van Leeuwenhoek 78: 399-405.

Letchumanan V, K Chan & L Le. 2014. *Vibrio parahaemolyticus*: A review on the pathogenesis, prevalence, and advance molecular identification techniques. Frontiers in Microbiology 5: 705.<doi:10.3389/fmicb.2014.00705>

Manimaran M, JV Gopal & K Kannabiran. 2017. Antibacterial activity of *Streptomyces* sp. VITMK1 isolated from mangrove soil of Pichavaram, Tamil Nadu, India. Proceedings of the National Academy of Sciences India, Section B 87(2): 499-506.

Mohan G, AK Thangappanpillai & B Ramasamy. 2016. Antimicrobial activities of secondary metabolites and phylogenetic study of sponge endosymbiotic bacteria, *Bacillus* sp. at Agatti Island, Lakshadweep Archipelago. Biotechnology Reports 11: 44-52.

Nonomura H. 1974. Key for classification and identification of 458 species of the *Streptomycetes* included in ISP. Journal of Fermentation Technology 52: 78-92.

Patil R, G Jeyasekaran & SA Shanmugam. 2001. Control of bacterial pathogens, associated with fish diseases, by antagonistic marine actinomycetes isolated from marine sediments. Indian Journal of Marine Sciences 30: 264-267.

Patil R, G Jeyasekaran & SA Shanmugam. 2016a. Incidence and activity of actinomycetes isolated from marine samples from Thoothukkudi, Tamil Nadu, on the east coast of India, against bacterial pathogens of fish. International Journal of Science and Nature 7: 541-549.

Patil R, G Jeyasekaran & SA Shanmugam. 2016b. Occurrence and antibacterial activity of actinomycetes isolated from marine samples from Thoothukkudi, Tamil Nadu, on the east coast of India, against fish pathogens. International Journal of Science and Nature 6: 338-347.

Pushpa-Rani KT & A Doss. 2016. Purification and antibacterial activity of marine actinomycetes against human and fish pathogens. Journal of Marine Science: Research and Develpoment 6: 4-7.

Roshan K, A Tarafdar, K Saurav, S Ali, SA Lone, S Pattnaik, A Tyagi, K Biswas & ZA Mir. 2013. Isolation and screening of bioactive compound from actinomycetes isolated from salt pan of Marakanam district of the state Tamil Nadu, India. Elixir International Journal 61: 16826-16831.

Santhakumari S, A Kannappan, SK Pandian, N Thajuddin, RB Rajendran & AV Ravi. 2016. Inhibitory effect of marine cyanobacterial extract on biofilm formation and virulence factor production of bacterial pathogens causing vibriosis in aquaculture. Journal of Applied Phycology 28: 313-324.

Selvakumar D, K Arun, S Suguna, D Kumar & K Dhevendaran. 2010. Bioactive potential of *Streptomyces* against fish and shellfish pathogens. Iranian Journal of Microbiology 2: 156-163.

Ser HL, UD Palanisamy, WF Yin, SNA Malek, KG Chan, BH Goh & LH Lee. 2015. Presence of antioxidative agent, Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-in newly isolated *Streptomyces mangrovisoli* sp. *nov.* Frontiers in Microbiology 6: 854. <doi: 10.3389/fmicb.2015.00854>

Sharma M. 2014. Actinomycetes: Source, identification, and their applications. International Journal of Current Microbiology and Applied Sciences 3: 801-832.

Sihag RC & P Sharma. 2012. Probiotics: The ecofriendly measure for sustainable development in aquaculture. Journal of Fisheries and Aquatic Sciences 7(2): 72-103.

Talavera G & J Castresana. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Systematic Biology 56: 564-577.

Thenmozhi M & K Kannabiran. 2011. Anti-*Aspergillus* activity of *Streptomyces* sp.VITSTK7 isolated from Bay of Bengal coast of Puducherry, India. Journal of Natural & Environmental Sciences 2: 1-8.

Thirumurugan D & R Vijayakumar. 2013. Exploitation of antibacterial compound producing marine actinobacteria against fish pathogens isolated from less explored environments. Asian Journal of Pharmaceutical Research 3: 75-78.

Thirumurugan D &R Vijayakumar. 2015. Characterization and structure elucidation of antibacterial compound of *Streptomyce*s sp. ECR77 Isolated from East Coast of India. Current Microbiology 70: 745-755.

Tuan NN, TT Trang, S Yaemkong, P Jaipong & P Kotham. 2017. Isolation and evaluation of antimicrobial activity of endophytic actinobacteria on May Chang tree (*Litsea cubeba*) against pathogenic bacteria causing diseases on common carp and tilapia. SNRU Journal of Science and Technology 9: 560-567.

Vaid N & ST Sajeevan. 2016. Screening marine bacterium strains of Actinomycetes as antagonists to pathogenic *Vibrios*, molecular identification of potent strain and media optimization by: Plackett-Burman analysis and response surface methodology. Invertis Journal of Science & Technology 9: 79-93.

Vaijayanthi, G, A Cholarajan & R Vijayakumar. 2012. Isolation, characterization and antibacterial activity of terrestrial actinobacteria in the soils of Thanjavur District, Tamil Nadu. Indian Journal of Science and Technology 4: 132-139.

Vijayakumar R & R Malathi. 2014. Isolation, characterization and antibacterial activity of actinobacteria from dye polluted soils of Tirupur. Facta Universitatis, Series: Medicine and Biology 16: 43-48.

Wang L, Y Chen, H Huan, Z Huang, H Chen & Z Shao. 2015. Isolation and identification of *Vibrio campbellii* as a bacterial pathogen for luminous vibriosis of *Litopenaeus vannamei*. Aquaculture Research 46: 395-404.

Williams ST, M Goodfellow & G Alderson. 1989. Genus *Streptomyces*. In: Williams ST, ME Sharpe & JG Holt (eds). Bergey's Manual of Systematic Bacteriology 4: 2452-2492.Williams & Wilkins, Baltimore.

Yang N & C Sun. 2016. The inhibition and resistance mechanisms of actinonin, isolated from marine *Streptomyces* sp. NHF165, against *Vibrio anguillarum*. Frontiers in Microbiology 7: 1-11. <doi: 10.3389/fmicb.2016.01467>

You JL, LX Cao, GF Liu, SN Zhou, HM Tan & YC Lin. 2005. Isolation and characterization of actinomycetes antagonistic to pathogenic *Vibrio* spp. from nearshore marine sediments. World Journal of Microbiology and Biotechnology 21: 679-682.

Zheng ZH, W Zeng, YJ Yang, ZY Huang, J Li, HR Cai & WJ Su. 2000. Detection of antitumor and antimicrobial activities in marine organism associated Actinomycetes isolated from the Taiwan Straint, China. FEMS Microbiology Letters 188: 87-91.

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