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ANTIBACTERIAL PROTEIN FRACTION DERIVED FROM *STREPTOMYCES FRADIAE* AGAINST SEPTICEMIA INFECTION IN *LABEO ROHITA*: BREAKTHROUGH IN MARINE DRUG DISCOVERY

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Abstract

The aquaculture sector continues to be an essential source of food, revenue, nutrition, and a means of subsistence for numerous people globally. Intensive aquaculture in a confined region induces environmental stress in farmed fish, which indirectly increases their vulnerability to many diseases. *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Vibrio harveyi* are the causative bacterial pathogens for septicemia, the most prevalent and commonly seen illness. Disease outbreaks result in a rise in mortality or a decrease in the quality of the meat, consequently inflicting substantial economic losses to carp production. In contrast, the continual use of antibiotics for infection management has contributed to the proliferation of drug-resistant bacteria in aquatic environments. In this context, marine actinobacteria are emerging as possible sources of new secondary metabolites to combat the problem. Actinobacteria are undoubtedly the most prolific producers of secondary metabolites, and they comprise several commercially and biotechnologically significant species. In this investigation, marine actinobacteria were isolated and evaluated for their antibacterial effectiveness against septicemia pathogens. Effective strains were identified as *Nocardiopsis* sp., *Streptomyces* sp., and *Pseudonocardia* sp. Candidate strains culture filtrates were treated with acetone to precipitate complete proteins. The antibacterial and anti-quorum sensing properties of the crude protein against *A. hydrophila* MTCC 1739 and *P. aeruginosa* MTCC 9425 were also examined using the agar well diffusion method. In addition, in vivo investigations of the low molecular weight fraction on L*abeo rohita* the fraction's efficacy in reducing septicemia infection. After the 8th day of bathing, fish were found to have recovered.

Keywords: Marine actinobacteria; *Aeromonas hydrophila*; Crude protein; Antibacterial activity; Anti-quorum sensing; Drug development

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1. Introduction

Aquaculture is the cultivation of freshwater and marine creatures, including fish, mollusks, crabs, and aquatic plants. Aquaculture refers to the practice of raising aquatic populations under controlled conditions. This method is distinct from commercial fishing, which relates to capturing wild fish. Over the past two decades, freshwater aquaculture has contributed over 95% of the overall aquaculture production in India, resulting in a six-and-a-half-fold increase in aquaculture. The three Indian major carps, namely Catla (*Catla catla*), Rohu (*Labeo rohita*), and Mrigal (*Cirrhinus mrigala*), account for nearly 1.8 million tonnes of output [1]. Despite the numerous socioeconomic benefits of aquaculture, its intensive development has led to several challenges in fish farming. The impact is determined to be twofold, harming aquaculture either by polluting the water or by causing disease outbreaks.

Septicemia infection is one of the most severe illnesses in the aquaculture industry. *A. hydrophila, P. aeruginosa,* and *V. harveyi* [2] are the pathogens responsible for the illness. Aeromonads are gram-negative facultative anaerobes that inhabit freshwater (such as groundwater and surface water), estuaries, and marine water habitats [3]. *A. hydrophila* is the most prevalent pathogen of warm-water fish, also infecting amphibians, reptiles, and humans [4]. In addition to hemorrhagic septicemia, the pathogen is responsible for illnesses such as dropsy, ulceration, asymptomatic septicemia, and exophthalmos in freshwater species [5], resulting in enormous economic losses in warm water aquaculture.

The pathogenicity of *A. hydrophila* is based on virulence factors and processes such as adhesions, toxins, proteases, hemolysins, motility, and biofilm formation [6]. Quorum sensing (QS) is a bacterial cell-to-cell communication system that regulates bacterial activity and physiological responses based on bacterial cell density. In *A. hydrophila*, the QS system is mostly dependent on the luxI/R homologous ahyI/R genes. Since quorum sensing (QS) plays a crucial role in bacterial pathogenesis, quorum sensing inhibitors (QSIs) derived from natural sources have attracted substantial interest for usage alone or in conjunction with medicines to prevent bacterial infection and progression of the disease [7].

Marine microorganisms continue to offer pharmacologically significant secondary metabolites that are distinct and new bioactive compounds that are being investigated for drug discovery. These compounds are created in response to stress, and many have promising biotechnological and medicinal uses. Burkholder [8] discovered and described the first antibiotic produced by the marine bacteria *Marina tinospora thermotolerans*.

A diversity of marine creatures has been removed from their natural habitats. Numerous actinobacterial species (*Streptomyces* and *Nocardiopsis*) are commonly found in marine habitats. *Streptomyces* is a prolific producer of secondary metabolites and produces more than 80 % of all commercially available antibiotics [9]. *Streptomyces* was previously reported to produce a variety of antimicrobial metabolites, including glycopeptides, beta-lactams, aminoglycosides, polyenes, polyketides, macrolides, actinomycin, and tetracycline. It has been revealed that marine *Streptomyces* sp. metabolites show anti-quorum sensing action. *Nocardiopsis* sp. is the most prevalent halophilic and halotolerant actinobacteria, according to a recent study [10]. It is known to create a variety of chemically distinct molecules with a broad spectrum of biological functions. In light of this, this research aims to isolate and identify potent actinobacteria from marine habitats and determine the effectiveness of protein metabolite against aquatic pathogenic bacteria.

2. Materials and methods

2.1 Sample collection

In the coastal region of Tamil Nadu, India, marine sediment samples were collected from Tuticorin, Thanjavur and Chennai in sterile polythene bags from 5 to 15 cm depth and two feet from the shoreline [11]. The samples were taken to the laboratory, where they were processed at 60 °C for 15 min in order to eliminate the moisture content and other bacterial contaminant before processing for isolation of actinobacteria.

2.2 Isolation of Marine actinobacteria

Marine actinobacteria were isolated using Starch Casein Nitrate (SCN) agar media [12]. One gram of marine sediment collected was suspended in 100 ml of sterile, distilled water, diluted serially, and 10 µl of each dilution from 10^{-2} to 10^{-5} was spread plated on SCN agar plates. Seven days were spent incubating the plates at 30 °C. Later, developing colonies of actinobacteria were selected morphologically and transferred to a fresh plate. For subsequent usage, the isolates were stored on SCN agar slants at 4 °C. Soil storage was carried out for permanent storage at -20 °C

2.3 Screening for antibacterial activity

The antibacterial activity of the isolated strains was initially determined by cross-streaking method [13] against septicaemia causing pathogens *A. hydrophila* MTCC-1739 and *P. aeruginosa* MTCC-9425 procured from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. The actinobacterial isolates were streaked in a linear pattern in the middle of SCN agar plates and incubated at 30°C. After six days, overnight cultures of test organisms were streaked perpendicular to the middle strip of the actinobacteria culture. The zone of inhibition was determined after re-incubating all plates at 37°C for 24 hours. For further research, potential strains were selected.

2.4 Isolation of protein from culture filtrate

Selected strains were cultivated for 10 days at 30˚ C in ISP 4 (International *Streptomyces* Project) broth. The cultivated media were centrifuged at 10,000 rpm to remove the cells, and the supernatants were filtered using Whatman No. 1 filter paper. To precipitate the protein from the culture filtrate, three times its volume acetone was added. This mixture was vortexed and kept at 4°C overnight. The suspension was centrifuged at 15000 rpm for 15 minutes. The precipitate was dried after the removal of supernatant, avoiding a complete dehydration of the protein pellet [14].

2.5 Estimation of total protein

The Bradford assay was done to estimate the total protein content in the precipitated sample. 20, 40, 60, 80 and 100µl of Bovine Serum Albumin (BSA) representing 20, 40, 60, 80 and 100µg respectively taken from a 1mg/ml stock solution were used as the standard. Fifty micro liters of the crude protein sample was taken and treated with Bradford reagent. The absorbance value of standard and sample was measured at 595 nm [15].

2.6 Antibacterial activity of the crude protein

The antibacterial activity of the crude protein precipitate was determined by the agar well diffusion method [16]. Mueller-Hinton Agar (MHA) plates were initially seeded with the overnight grown culture of test organisms *A. hydrophila* MTCC-1739 and *P. aeruginosa* MTCC-9425. Wells were punctured on the seeded plates (MHA) with the help of a sterile cork borer. Fifty microliters of the crude protein (1mg/ml), Tris buffer (negative control to eliminate any antagonistic effect of the buffer) and kanamycin ((1 mg/ml) which served as positive control) were added to the respective wells. The diameter of the inhibition zone around the well was measured after 24 h of incubation at 37 °C. Experiments were done in triplicates and the results were expressed in mean value and standard error.

2.7 Identification of Actinobacteria

2.7.1 Phenotypic Characterization

Examining the cultural characteristics and spore morphology of potential isolates was done to assume the identity of these isolates [17].

2.7.1.1 Cultural characterization of SCNA medium

Initially cultural characterization of all the seven potential strains was done on SCN agar. Plates were inoculated and incubated at 30°C for 7-14 D. After incubation, plates were observed for macroscopic characters such as colony appearance, color of ariel mycelium as well as substrate

mycelium, reverse-side pigmentation, presence of melanin pigments and glycerol globules.

2.7.1.2 Spore morphology

The spore morphology is viewed by slide culture technique [18]. All seven potential strains were grown on SCN agar medium. Sterilized microscopic slides were taken and a 1-2 mL of medium was poured on each slide. After solidification, the seven potent strains were patched on the medium individually. The inoculated slides were placed in the moist chamber and incubated till sporulation at 30°C. Later, slides were observed for microscopic characteristics such as the arrangement of spores, shape of spores and their ornamentation [19].

Arrangement of spores on mycelium was also observed under SEM (Scanning Electron Microscope). Sequential fixation of microbial samples transferred to glass cover slips using percentages of ethanol (25 % to 100 %) was done. The fixed specimens were coated with gold and observed in high vacuum mode.

2.7.1.3 Cultural characterization of ISP media

The cultural characterization for the five potential strains assumed to be genus *Streptomyces* based on the spore arrangement was done on ISP 1-7 media [20]. Plates were inoculated and incubated at 30°C for 7-14 D. Morphology of aerial and substrate mycelium production of diffusible pigments was detected in ISP 2, 3, 4 and 5 media. Production of melanin pigment and glycerol globules was observed in ISP 6 and 7 media. The exhibited colour pattern of the aerial mycelium was compared with colour wheel reported by Tresner and Backus [21] to identify the species.

2.7.2 Biochemical characterization

Selected seven isolates were further analyzed for their biochemical characteristics through MR-VP, catalase, oxidase, citrate utilization, nitrate reduction, Triple Sugar Iron (TSI) Agar, and enzymes hydrolysis activity for amylase, gelatinase, esterase, caseinase and urease. Ability to decompose tyrosine, cellulose and pectin was also analyzed. Cultures were grown in respect media and incubated for seven days at 30°C. Results were noted and compared with Bergey's Manual [22] to determine the identity of the isolates.

2.7.3 Molecular characterization

16S rRNA gene sequencing was performed at Macrogen Korea, with the universal forward primer 785F 5' (GGA TTA GAT ACC CTG GTA) 3'and reverse primer 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'. PCR amplification of the template DNA was done. Amplified nucleotide was sequenced and matched using the BLAST program. The phylogenetic tree was constructed using the neighbor-joining method. Sequences of the isolates were submitted to Gen Bank and accession numbers were obtained.

2.8 Detection of quorum sensing inhibition by crude protein

Quorum sensing inhibition by crude protein was determined using agar well diffusion method [23]. *C. Violaceum* MTCC-2656 was lawned continuously in three directions onto Luria - Bertani (LB) agar using sterile cotton buds. Then, a sterile cork borer was used to puncture wells on the inoculated plates, 50µl of the crude protein precipitate (1mg/ml) and Streptomycin ((1 mg/ml) positive control) were added to the respective wells. The plates were incubated at 37°C for 24 h. After that, plates were observed for inhibition of the violacein pigment around the well.

2.9 Size Exclusion chromatography

Separation of active protein from crude protein of the potential strain *S. fradiae* TPM was done according to the method described by Zielinska [24]. Crude protein of potent isolate TPM (500 mg) was passed through Sephadex G10 column (Sigma Aldrich, USA), (column: 1.5 × 30 cm) using 50 mM Tris-HCl (pH 8.5) as mobile phase. Volumes of two milliliters per tube of the eluent were collected and all the collected fractions were analyzed for their antibacterial activity against *A. hydrophila* MTCC-1739 and *P. aeruginosa* MTCC 9425. Fractions showing the highest activity were further subjected to SDS- PAGE and estimated using Bradford assay [15].

2.10 Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis

The SDS-PAGE technique was employed to determine the approximate size of the active protein. The protein samples were prepared using Laemmli sample buffer. .20 µl of fractionated protein sample was loaded on 4% stacking polyacrylamide gel over 15% polyacrylamide separation gel. Protein molecular marker with the range of 40 -1.7 K Da (Thermo Fisher Scientific Ltd.) was used. The gel was electrophoresed at a constant current of 20 mA in a Bio-Rad vertical electrophoresis tank until the dye reached 3/4th of the gel. The SDS- PAGE gel was stained with Coomassie Blue R-250 solution for 1 h at room temperature. Afterward, the gel was immersed in a de-staining solution (3% methanol, 1% acetic acid) at room temperature until complete disappearance of the background blue color. The gel images were captured using a gel document system Image Quant LAS 500 [25].

2.11 *In vivo* studies

2.11.1 Test pathogen

Septicemia pathogen A. hydrophila MTCC 1739 was used for this study. The culture was maintained on a nutrient agar slant (Hi media, Mumbai) at 8°C and routinely tested for its virulence on Blood Agar (BA). A stock culture in 70% glycerol (v/v) was stored at -20°C to provide stable inoculum throughout the study [26, 27]. Overnight culture of *A. hydrophila* MTCC 1739 was raised from the glycerol stock on tryptone soy agar (Hi Media) and harvested in tryptone soy broth. The culture was diluted with fresh tryptone soy broth during the logarithmic growth phase to an OD of 0.1 (corresponding to 10^9CFU/mL). This culture was used as the inoculum for inducing infection in *rohu* [28].

2.11.2 Physicochemical analysis

Physicochemical parameters such as Total alkalinity, pH, total dissolved oxygen (DO), total hardness (TH), Salinity and BOD, along with the concentration of calcium (Ca^{2+}), nitrate (NO^{3-}), nitrite (NO^{2-}), ammonium (NH^{4+}), phosphorus (P), Iron (Fe), fluoride (F) chloride (Cl^-), Residual chlorine were determined using Nice water quality kit. All the values were compared with standard water quality values provided with the kit.

2.11.3 Experimental animals

The carp, *Labeo rohita* (average length = 10 ± 2 cm), was procured from Tamil Nadu Fisheries Department in Bhavanisagar, Tamil Nadu. The fishes were transported in stock tanks filled with oxygenated water and were acclimatized to the laboratory environment in a cement tank (capacity 2000

L; 6×4×4 m) for 3 weeks under normal conditions (23 ± 2°C). The water quality was checked initially as mentioned above and was maintained as per the requirement for the survival of the animals. Fishes were fed twice a day with commercial fish feed throughout the period of the study and the bottom of the tank was cleaned daily by siphoning out the waste material along with a partial exchange (10 %) of fresh water [29].

2.11.4 Induction of infection in *L. rohita*

A. hydrophila MTCC 1739 was injected intramuscularly using a 3/10-cc U-100 ultrafine insulin syringe with a 0.5-inch-long (ca. 1-cm-long) 29-gauge needle (catalog no. BD-309301; VWR) holding 10 µL of the bacterial suspension. During the process, care was taken not to injure the experimental animals and the procedure was followed as per ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines developed as part of a National Centre for the Replacement, Refinement and Reduction of Animals in Research. Fishes that developed lesions were used in the experiment.

2.11.5 Preparation of the treatment tanks

Ten milligrams of the crude protein isolated from the potent strain *S. fradiae* TPM was dissolved in 5 mL of 50mM Tris-HCl of pH 8.5. The treatment tanks were prepared for one litre with 5 mL of dissolved active crude protein to achieve a final concentration of 10 mg/L. This prepared tank was used for bath treatment of the Group III animals. In a similar manner treatment tanks for 14.4 KDa antibacterial protein from *S. fradiae* TPM and commercial antibiotic kanamycin were prepared for Group IV and V animals. All the treatment tanks were prepared freshly every day before treatment.

2.11.6 Experimental design

The customized fishes were divided into five groups with six fishes. The experiment was conducted in the following design.

Group I (Control): Fishes injected with 10µL of distilled water.

Group II (Untreated): Fishes injected with test pathogen kept without treatment.

Group III (Treated with crude protein): Fishes injected with test pathogen and treated with the crude protein of *S. fradiae TPM.*

Group IV (Treated with low molecular weight protein): Fishes injected with test pathogen and treated with the low molecular weight protein isolated from *S. fradiae* TPM.

Group V (Treated with Commercial antibiotic): Fishes injected with test pathogen and treated with the commercial antibiotic Kanamycin.

2.11.7 Treatment

Fishes with a hemorrhagic spot that progressed to form an epidermal lesion were taken for treatment. After the development of the lesion with subsequent swelling, infected fishes of groups III, IV and V were allowed to bath for 1 h/D every day in their respective treatment tank until the swelling and lesion becomes in significant. The size of the lesions was measured (in cm) 24 h after treatment. Feed intake and activity of the fish was also monitored. The data was statistically analyzed.

3. Results

3.1 Collection of marine soil sediment samples

Twenty-three marine sediment samples were collected from the coastal region of Tamil Nadu. The details of sediment collected are given in the Table 1.

Table 1 Details of marine soil sediment samples

Location		Places of Collection	Soil Code	No. of Sample
State	District			
Tamil Nadu	Tuticorin	Harbour (N)	HN	1
		Harbour (S)	HS	1
		Muyal Island	MI	1
		Kulasekara Pattinam beach	KP	1
Tamil Nadu	Thanjavur	Malipattinam	Mm	1
		Chinnamalai	CI	1
		Velivayal	VL	1
		Pudhupattinam	TPM	1
Tamil Nadu	Chennai	Marina beach	MB	2
		Kovalam	KOM	1
		ECR (Panayoor) beach	ECR	1
		Kottivakkam	KM	1
		Thirumudivakkam	THM	1
		Sandhom	SM	1
		Gandhi Nagar beach	GNB	1
		Pattinampakkam	PPM	1
		Thiruvanmaiyur	THR	1
		Palavakkam	PAM	1
		Besant Nagar beach	BNB	1
		Besant Nagar(E)	BNE	1
		Mahabalipuram	MAM	1
		Pazhaverkadu	PUV	1
Total				**23**

3.2 Isolation of marine action bacteria

Totally thirty-one strains were isolated from the marine sediment samples collected. Details of the isolates obtained are listed in Table 2. All the isolates obtained were named with letters representing their sampling area.

Table 2 List of marine action bacteria isolated

Location	Place of collection	Total No. of isolates	Name of isolates
Tuticorin	Harbour (N)	1	THN1
	Harbour (S)	-	-
	Muyal Island	-	-
	Kulaserkarapattinam	2	KP1, KP2
Thanjavur	Pudhupattinam	1	TPM
	Malipattinam	-	-
	Chinnamalai	-	-
	Velivayal	-	-
Chennai	Marina beach	1	MB1
	Kovalam	4	KOM1, KOM2, KOM3, KOM4
	Kottivakkam	1	KM1
	ECR(Pananyoor)	7	ECR1, ECR2,

beach		ECR3, ECR4, ECR5, ECR6, ECR7
Thirumudivakkam	3	THM1, THM2, THM3
Santhom	2	SM1, SM2
Palavakkam	1	PAM1
Pattinampakkam	7	PPM1, PPM2.PPM3, PPM4, PPM5, PPM6, PPM7
Pazhaverkadu	1	PVU
Thiruvanmaiyur	-	-
Mahabalipuram	-	-
Besant Nagar beach	-	-
Besant Nagar(E)	-	-
Gandhi Nagar beach	-	-
Total	**31**	

3.3 Screening for antibacterial activity

Among the 31 isolates of marine actinobacteria tested 14 strains showed antibacterial activity against septicemia pathogens *A.hydrophila* MTCC-1739 and *P. aeruginosa* MTCC-9425 (Table 3). Based on the primary screening, these 14 strains KP2, TPM, KOM2, KOM4, ECR1, ECR4, ECR16, THM2, SM2, PAM1, PPM1, PPM5, PPM7 and PVU were selected for further studies.

Table 3 Primary screening of antibiotic producing isolates

S.NO	Isolated strains	*A. hydrophila* MTCC- 1739	*P. aeruginosa* MTCC-9425
1	THN1	-	-
2	KP1	-	-
3	KP2	+	+
4	TPM	+	+
5	MB1	-	-
6	KOM1	-	-
7	KOM2	+	+
8	KOM3	-	-
9	KOM4	+	+
10	KM1	-	-
11	ECR1	+	+
12	ECR2	-	-
13	ECR3	-	-
14	ECR4	+	+
15	ECR5	-	-
16	ECR16	+	+
17	ECR7	-	-
18	THM1	-	-
19	THM2	+	+
20	THM3	-	-
21	SM1	-	-
22	SM2	+	+
23	PAM1	+	+
24	PPM1	-	-
25	PPM2	+	+
26	PPM3	-	-
27	PPM4	-	-
28	PPM5	+	+
29	PPM6	-	-
30	PPM7	+	+
31	PVU	+	+

Positive (+), Negative (-)

3.4 Crude protein precipitation

The selected 14 isolates were subjected to crude protein precipitation using acetone. The concentration of protein in the sample was also determined by the Bradford assay (Table 4).

Table 4 Concentration of precipitated protein

Name of the isolates	Concentration of crude protein µg/ml
PPM7	26.73
KOM4	48.22
KP2	43.54
TPM	44.71
ECR1	28.11
ECR16	25.56
SM2	32.69

3.5 Antibacterial activity of the crude protein

In the secondary screening, crude protein of seven potent strains showed activity in agar well diffusion assay. The crude protein of the isolates KP2, TPM, PPM7 and KOM4 exhibited prominent activity with a wide zone of inhibition than ECR1 & ECR16 against *A. hydrophila* MTCC-1739 and *P. aeruginosa* MTCC-9425. Crude protein of the strain SM2 showed maximum zone of inhibition among the seven against *P. aeruginosa* MTCC-9425 (Fig 1).

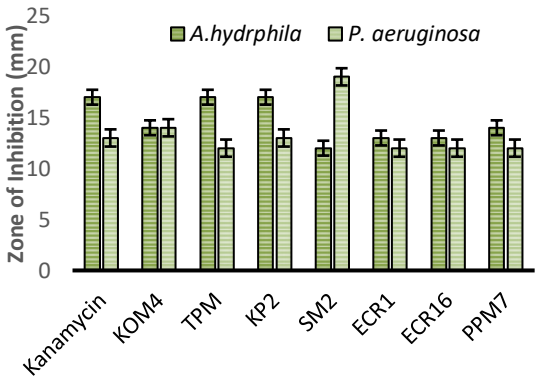


Fig. 1. Antibacterial activity of precipitated protein

3.6 Characterization of potent strains

3.6.1 Phenotypic characterization

3.6.1.1 Cultural Characterization on SCNA medium

Cultural characteristics of seven potent strains were examined on SCN agar medium initially. The isolated strains KP2, TPM showed luxurious growth with yellow-brown +red type of substrate mycelium and pink-coloured aerial mycelium possessing a powdery appearance and rough nature. Isolates ECR16, ECR1and SM2 showed luxurious growth and aerial mycelium was gray in color with powdery nature. Substrate mycelium of ECR1and SM2 belonged to the yellow-brown group, while strain ECR16 exhibited yellow coloured substrate mycelium. The substrate mycelium of strain KOM4 was deep-brown coloured and penetrated the agar. The diffusible pigment produced was yellowish brown in colour, which was observed on the agar plate. The aerial mycelia showed luxurious growth, white colour with a mild pimento touch. The isolate PPM7 showed luxurious growth with substrate mycelium being creamy white coloured and aerial mycelium white coloured. The details are listed in Table 5, Fig 2.

Table 5 Cultural characterization of potent isolates on SCNA medium

S.NO	Potent isolates	Medium	Characteristics			
			Spore Mass	Pigmented	Aerial mycelium	Substrate mycelium
1	KP2	SCNA (Starch Casein Nitrate Agar)	+++	-	Pink	Yellow- Brown+ Red
2	TPM		+++	-	Pink	Yellow-Brown+ Red
3	KOM4		+++	Diffusible pigment (Yellowish brown)	White	Brown
4	PPM7		+++	-	White	Creamy white
5	ECR1		+++	-	Gray	Yellow- Brown
6	ECR16		+++	-	Gray	Yellow
7	SM2		+++	-	Pink	Yellow- Brown

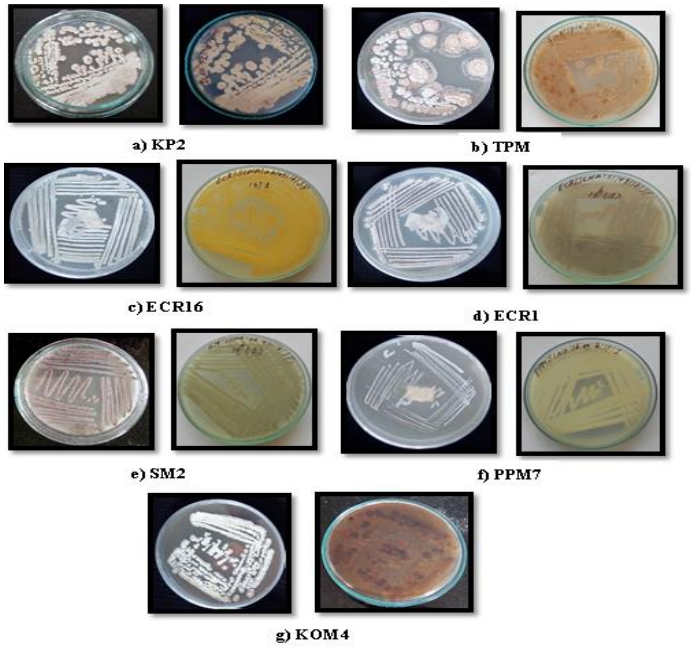


Fig 2 Cultural characterization of potent isolates on SCNA medium

3.6.1.2 Spore morphology of potent isolated strains

Types of spore chain and morphology of the spores of all the select isolates were observed under light microscope and Scanning Electron Microscope (SEM). Spore-bearing hyphae of isolates ECR1, ECR16 and SM2 was observed to be verticillate type possessing spiny spore (Fig 3 c, d and e). Isolates KP2 and TPM had retinacutiperti type on sporophore bearing smooth spores (Fig 3 a and b). As these types of spore arrangement are seen only in the genus *Streptomyces* [20], these five isolates were assumed to belong to *Streptomyces*.

Isolate KOM4 beared disjointed spores on branched aerial hyphae, a characteristic feature of the genus *Nocardiopsis*. Spores featured on the different sporophores are wrapped together to form synnemata. Due to synnemata's presence, this isolate was identified to belong to the genus *Nocardiopsis* species *synnemataformans* (Fig 3 f).

Longitudinal spores, either singly or in pairs seen on long vegetative hyphae that get fragmented, are characteristic features of the genus *Pseudonocardia*. As similar spore type was observed in isolate PPM7, it was assumed that the isolate belongs to *Pseudonocardia* (Fig 3 g).

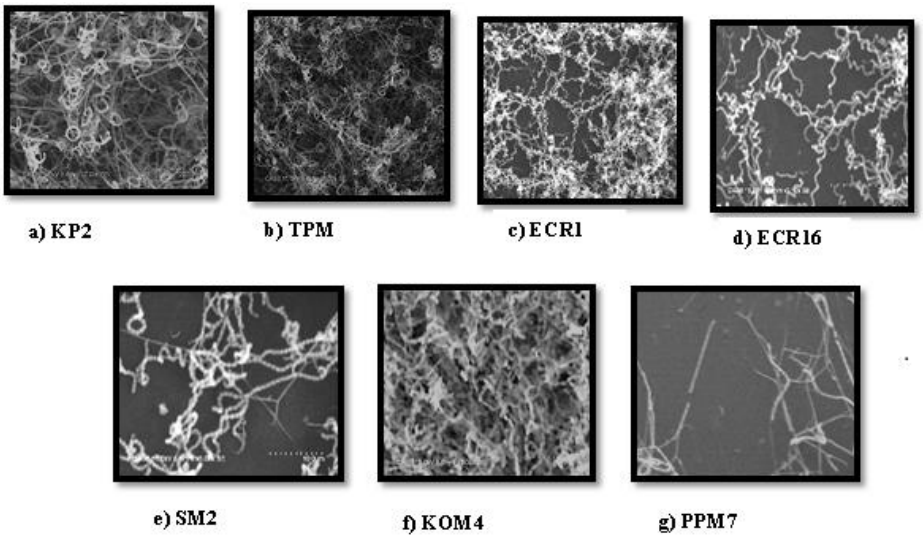


Fig. 3. Spore and spore chain morphology of potent strains

3.6.1.3 Cultural Characterization on ISP 1-7 media

The isolates KP2, TPM, ECR16, ECR1and SM2, which were predicted to belong to the genus *Streptomyces* based on their arrangement of spores on the sporophore, were taken for further characterization of their colony morphology on the set of ISP media. The isolated strains KP2, TPM showed luxurious growth in ISP3 and ISP4, while moderate growth was observed in ISP2. Aerial mycelium was pink colour with powdery and rough nature in all three media. Substrate mycelium belonged to yellow-brown + red group as classified by Szabo and Marton [30]. No soluble or diffusible pigment production was observed in ISP5 medium. No melanin production on ISP6 and ISP7 medium was seen. As per the earlier report by Tresner and Backus [21] presence of pink colour in the aerial mycelium of TPM and KP2 suggested them to be *Streptomyces fradiae* (Table 6).

Aerial spore mass of isolates ECR16, ECR1 and SM2 appeared gray colour in ISP 2,3,4 media. Substrate mycelium of these isolates belonged to the basic yellow-brown group with a slight difference in the intensity of yellow colour. No diffusible pigment or melanoid production was observed with all these isolates on ISP6 and 7 media, these isolates were identified to belong to the species *werraensis* (Table 6).

Table 6 Cultural characterization of selected isolates on various ISP media

Media	Isolates				
	KP2	**TPM**	**ECR16**	**ECR1**	**SM2**
Growth	+++	+++	+++	+++	+++
Aerial Mass Colour					
a. ISP 2	Pink	Pink	Gray	Gray	Gray
b. ISP 3	Pale Pink	Pale Pink	Gray	Gray	Gray
c. ISP 4	Pale Pink	Pale Pink	Dark Gray	Dark Gray	Dark Gray
Reverse Colour					
a. ISP5	Yellow-Brown+ Red	Yellow-Brown+ Red	Yellow - Brown	Yellow- Brown	Yellow- Brown
Diffusible Pigment	-	-	-	-	-
Melanin Production					
a. ISP6	-	-	-	-	-
b. ISP7	-	-	-	-	-

Growth Rate: (+++)-luxurious, (++) – Good, (+) – Poor (moderate)

3.6.2 Biochemical characterization

Biochemical characterization of strain KOM4 showed positive reactions to methyl red, hydrolysis activity of catalase, amylase, protease, urease, and tyrosinase. While others were negative. In comparison with Bergey's manual [18], strain KOM4 belong to the genus *Nocardiopsis* (Table 7).

The strain PPM7 showed a positive reaction for citrate utilization, amylase, protease, tyrosinase, oxidase, catalase, urease, nitrate reduction and gelatinase. Indicating that this isolate belongs to the genus *Pseudonocardia* Bergey's manual [18] (Table 7). Biochemical characterization of the isolates KP2, TPM, ECR1, ECR16 and SM2 showed positive reactions to methyl red (variable), Voges Proskauer, citrate, amylase, protease, tyrosinase, catalase, oxidase, urease, nitrate reduction (variable) and gelatinase. They were able to produce gas and acid butt on TSI (variable). The above pattern of biochemical characteristics exhibited by these five isolates showed that they belong to the genus *Streptomyces* (Table 7).

Table 7 Biochemical characteristics of the potent strains

Character	TPM	KP2	SM2	ECR1	ECR16	KOM4	PPM7
Biochemical Characterization							
Methyl Red test	+	+	-	-	-	+	-
Indole Production	-	-	-	-	-	-	-
VP	+	+	+	+	+	-	-
Citrate Utilization	+	+	+	+	+	-	+
Amylase Production	+	+	+	+	+	+	+
Protease Production	+	+	+	+	+	+	+
Esterase Production	-	-	-	-	-	-	-
Tyrosine Degradation	+	+	+	+	+	+	+
Pectin Degradation	-	-	-	-	-	-	-
Catalase Production	+	+	+	+	+	+	+
Oxidase Production	+	+	+	+	+	-	+
Urease	+	+	+	+	+	+	+
Hydrogen Sulfide Production	-	-	-	-	-	-	-
Nitrate Reduction	+	+	-	-	-	-	+
Gelatin liquefaction	+	+	+	+	+	-	+
TSI	+ A/L	+ A/L	+	+	+	-	-

Positive (+), Negative (-), AL/AL- Alkaline slant and butt

3.6.3 Molecular characterization of potent strains

The 16S rRNA gene sequence of the isolates was generated and compared with the nucleotide sequences of other action bacterial strains retrieved from the NCBI Gen Bank database. The isolates KP2, TPM showed maximum homology (99%) with *Streptomyces fradiae*, strain KOM4 showed 99% of *Nocardiopsis synnemataformens,* strain PPM7 showed 98.97% of similarity with *Pseudonocardia xishanensis* and isolates SM2, ECR1, and ECR16 showed 100% similarity with *Streptomyces werraensis*.

The sequence of isolates KP2, TPM, KOM4, PPM7 and SM2 were submitted to the Gene bank (NCBI, USA) and accession number were obtained as *Streptomyces fradiae* MG748835, *Streptomyces fradiae* MG263520, *Nocardiopsis synnemataformans* MG280925, *Pseudonocardia xishanensis* MG280926 and *Streptomyces werraensis* MG748836 respectively.

The morphological, biochemical and molecular characterization results confirmed that the isolates KP2, TPM belongs to *Streptomyces fradiae,* isolate KOM4 belong to *Nocardiopsis synnemataformans*, isolate PPM7 belongs to *Pseudonocardia xishanensis* and isolates SM2, ECR1, ECR16 belongs to *Streptomyces werraensis.*

3.7 Detection of anti-quorum sensing activity by crude protein

The crude protein was tested for QS inhibition using indicator organism *C. Violaceum* MTCC- 2656. Results obtained showed inhibition of the violacein pigment around the well, without affecting the bacterial growth (Fig. 4a, b, c) by isolates KP2, TPM and KOM4 crude protein indicating it had significant QS inhibition activity. Crude protein from other four isolates showed no inhibition.

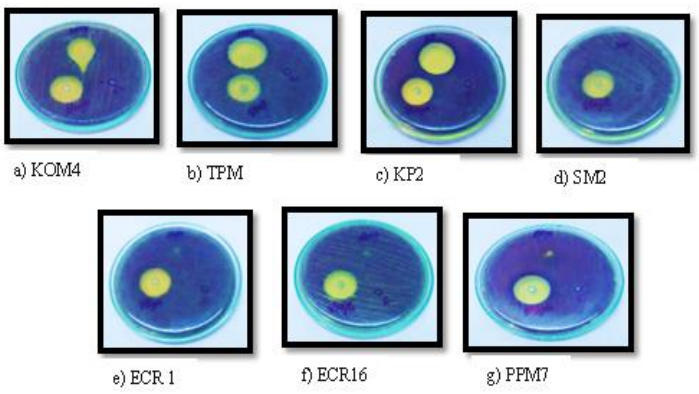


Fig. 4. Detection of anti-quorum sensing activity by crude protein

3.8 Size Exclusion Chromatography

Size exclusion chromatography on Sephadex G10 (SEC) was performed to separate low molecular weight active proteins from the crude. Totally 35 fractions were collected and first 30 fractions showed antibacterial activity against *A. hydrophila* MTCC-1739 and *P. aeruginosa* MTCC 9425 (Fig. 5).

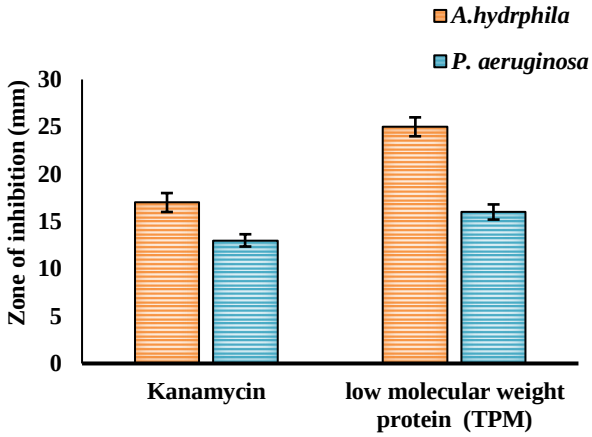
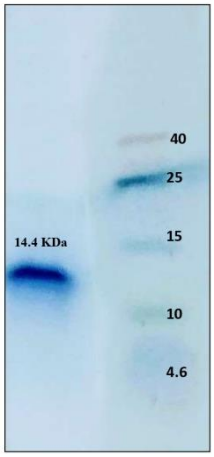


Fig. 5. Antibacterial activity of low molecular weight protein

3.9 SDS-PAGE

The result of collected fractions by SEC run on 15% SDS-PAGE is shown in Fig 6. The fractions possessed a single protein molecule with a molecular weight 14.4 KDa. All the active fractions were pooled together and lyophilized.



Lane1- Low molecular weight protein
Lane 2- Marker

Fig. 6. Determination of molecular weight by SDS – PAGE

3.10 Estimation of fractionated protein

The concentration of the purified protein was estimated to be 76.734 µg/ml in the prepared stock sample.

3.11 *In vivo* study

3.11.1 Physicochemical analysis

Physicochemical parameters act as a vital role in the aquaculture ecosystem. The optimal water condition has to be provided for the cultivation of fish. Hence physicochemical parameter of the tank was determined. All the parameter was within the standard range showing that the water is ideal for cultivation. The results of this study are presented in Table 8.

Table 8 Physicochemical analysis

S. No	Particular	Standard Values	Experimental tank
1.	pH	7 - 8.5	7 ± 0.5
2.	Total alkalinity	50-300 ppm	140 ppm
3.	Dissolved oxygen (DO)	<4.0 mg/L	3.5 mg/L
4.	Hardness	40-400 ppm	250 ppm
5.	Calcium (Ca2+)	1-135 mg/L	100 mg/L

6.	Nitrate (NO3 -)	<100 mg/L	20 mg/L
7.	Nitrite (NO2 -)	<1 ppm	0.0 ppm
8.	Ammonium (NH4+)	0.5 ppm	0.2 ppm
9.	Phosphorus (P)	0.02 ppm	0.0 ppm
10.	Iron (Fe_2O_3)	0.3 ppm	0.0 ppm
11.	Fluoride (F)	0.5- 1.5 mg/L	1.0 mg/L
12.	Chloride (Cl-)	1.5-2.0 ppm	1.75 ppm
13.	Residual chlorine	10 -50 mg/L	5.34 mg/L
14.	Salinity	15-25 ppt	17 ppt
15.	BOD	<50 mg/L	30 mg/L

3.11.2 Clinical signs

Five days after injection, the infected fish showed increased respiration and lethargy. Some fishes showed a marked hemorrhage at the base of the fins and the vent. The skin lesions, which initially appeared as a white discoloration, developed to shallow hemorrhagic ulcers at the point of injection.

3.11.3.1 Progress of infection in group-II

The lesion area of group II animals was found to increase in size from 0.7±0.01cm on 5thD to 5.5±0.01 cm on the 10th D. Ulceration commenced as sloughing-off of the scales, followed by the occurrence of a hemorrhagic spot that progressed to form an epidermal lesion. The lesion expanded daily and affected the internal muscles, indicated by the dark (blackish-red color) hemorrhage spots observed in the infected area. Death of the infected fishes was observed from 7th day (Fig.7.e).

3.11.3.2 Effect of treatment with *S. fradiae* TPM crude protein extract

In group III, the lesion area got reduced in size from 0.5 ±0.01 cm to 0.1±0.01 cm after 10th D of bath treatment (Fig. 7. a, and b). Swelling area subsided after 4th D of treatment.

3.11.3.3 Effect of treatment with active protein from *S. fradiae* TPM

Group IV animals treated with active protein from *S. fradiae* TPM showed a reduction in the lesion size from 0.7 ±0.01cm to 0.1±0.01 cm after 8th D of treatment (Fig.7.c). A decrease in the swelling area was observed, which completely subsided after 3rd D of treatment. The fishes recovered from infection after eight days of treatment.

3.11.3.4 Effect of treatment with commercial antibiotic

Group V animals treated with the commercial antibiotic kanamycin showed a reduction in the lesion size from 0.5 ±0.01cm to 0.1± 0.01cm after the 5th D of treatment. A decrease in the swelling area was also observed, which completely subsided after 3rd D of treatment. The fishes recovered from infection after five days of treatment (Fig.7.d).

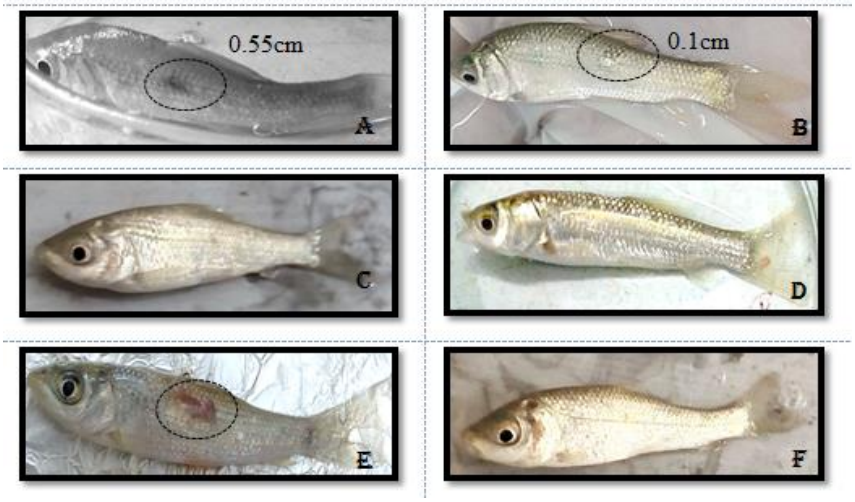


***Fig.* 7. Treatment of septicemia infection**

A) Before treatment, B) After 10th day treatment (Crude protein TPM), C) After 8th day treatment (Low molecular weight protein), D) After 5th day treatment (Kanamycin), E) Untreated 10th day, F) Control

4. Discussion

Aquaculture is emerging as the fastest growing food-producing industry in the world because of the increasing demand for food fish consumption. To combat the increasing need by the consumers, composite cultivation has developed to grow two or more non-harmful species of Indian major carps like Catla (*Catla catla*), Rohu (*Labeo rohita*), and Mrigal (*Cirrhinus mrigala*) together. Such intensive culture of edible fish has led to outbreaks of bacterial diseases. Bacterial hemorrhagic septicemia caused by *A. hydrophila, P. aeruginosa* and *V. harveyi* is one of the most devastating bacterial diseases known, resulting in annual economic losses to the aquaculture industry estimated at billions of dollars worldwide. Among the three causative organisms *A. hydrophila* is known to play a major role.

The present study was focused on isolating marine actinobacteria from sediments collected in the coastal regions and analyzing the antibacterial activity of metabolites

produced by the selected isolates against *A. hydrophila* MTCC - 1739 and *P. aeruginosa* MTCC-9425. Marine organisms are recently reported to produce novel secondary metabolites to combat their environmental conditions. Such metabolites have immense market value as antibiotics, anticancer compounds, etc. [31, 32]. A total of 31 strains were isolated and most were observed to produce pigments. All the isolated isolates were screened for their antibacterial activity against the *A. hydrophila* MTCC-1739 and *P. aeruginosa* MTCC-9425. Among the 31 isolate 14 were found to be active against the test pathogens. Earlier studies have reported the production of antibacterial compounds from marine actinobacteria. Anticandidal protein and antimicrobial compound from *N. dassonvillei* MAD08 a sponge-associated marine actinobacterium have been reported by Selvin [33]. The antifungal compound was also identified from *Streptomyces sp*. VSBT-501isolated from marine sediments, which was found to be effective against Candida albicans, *Penicillium chrysogenum*, *Aspergillus flavus, A. niger, A. fumigatus, Geotrichum candidum* and *Saccharomyces cerevisiae* [34].

Earlier reports are available for the presence of antibacterial compound in marine Actinobacteria against fish pathogens that support our findings. Thirumurugan *et al.,* [35] reported the presence of antibacterial compound methyl-4,8-dimethylundecanate from *Streptomyces albogriseolus* ECR64 isolated from marine sediments, to be effective *against Vibrio cholerae, V. parahaemolyticus, V. alginolyticus, Pseudomonas fluorescens* and *Aeromonas hydrophila*. The antibacterial and fungal compounds phenol, 2,4-bis (1,1 dimethylethyl) was identified from *Streptomyces sp*. MK388207 isolated from marine sediments, which was found to be effective against *Aeromonas hydrophila*, *Vibrio damsela*, *Staphylococcus aureus* ATCC6538, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 19404 and *Candida albicans* ATCC 10231[36]. Mondal and Thomas [37] has also reported antibacterial compound from *Beijerinickia fluminensis* VIT01 marine actinobacteria against fish pathogens.

As our interest was with identifying antibacterial protein producing isolates, we identified seven potent isolates (KP2, TPM, ECR1, ECR16 and SM2) that produced an antibacterial protein effective against septicemia pathogens using agar well diffusion assay. Studies have so far reported the identification of active protein from marine actinobacteria. Notably, genus *Streptomyces* and *Nocardiopsis* belonging to marine ecosystem have been reported earlier to produce polypeptide, thiopeptide and glycopeptide that were effective against streptomycin and vancomycin-resistant bacteria [38], [39],[40]. Protein from marine actinobacteria were reported to be effective against human pathogens like Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Proteus mirabilis, P. vulgaris, Escherichia coli, Bacillus subtilis, B. megaterium, B. cereus, B. sphereus, B. sterothermophilus, Micrococcus luteus, Pseudomonas putida, P. aeruginosa, Salmonella typhi* and *S. paratyphi*. Our study evaluated the potency of antibacterial protein isolated from marine actinobacteria to inhibit *A. hydrophila* MTCC-1739 and *P. aeruginosa* MTCC-9425.

Identification of these potential isolates becomes an unavoidable part. Hence the phenotypic, microscopic and biochemical characters were studied.

Isolate KOM4 was observed to grow luxuriously in SCNA medium. Aerial mycelium was white coloured with a brown-colored substrate mycelium that penetrated into the agar. Spores observed on a sporophore are a fragmenting hyphal type wrapped together in a synnemata. The strain was citrate positive and hydrolyzed casein, tyrosine and urea. Production of diffusible pigment was also observed. These observed characteristics suggested that the isolate belongs to genus *Nocardiopsis* and species *synnemataformans* as indicated in Bergy's manual vol 5 [22]. The 16S rRNA gene sequence also confirmed its identification.

Isolate PPM7 exhibited the characteristic features of genus *Pseudonocardia,* such as mycelium fragmentation and longitudinal single rectangular spore on the aerial mycellium. There was no production of diffusible pigment in the cultured plates. They were able to utilize starch, nitrate and also urea as reported in Bergy's manual. Later, 16S rRNA gene sequence also confirmed the isolate PPM7 as *Pseudonocardia xishanensis.* Zhao *et al*., [41] reported a similar observation with endophytic *Pseudonocardia xishanensis,* which was isolated from roots of *Artemisiaannua* L.

Genus *Streptomyces* was known to have a colony morphology of discrete or lichenoid with a long chain of spore arranged as retinacutiperti, verticillate bearned on as open hook. They are also reported for the utilization of different substrates such as casein, gelatin, starch, nitrate and tyrosine. Most species are said to produce pigments on aerial hyphae. Five among the seven isolates selected for antibacterial activity in this study KP2, TPM, ECR1, ECR16 and SM2 possessed the above reported characters and hence were assumed to belong to the genus *Streptomyces*.

From the above five *Streptomyces* isolates KP2 and TPM showed production of sea shell pink pigment on the aerial mycelium. Based on the colour of this pigment as per the report of Tresner and Backus [30], these isolates were identified to belong to the species *fradiae.* 16S rRNA gene sequencing also supported our identification.

The other three isolates ECR1, ECR16 and SM2 showed a gray colour pigmentation with verticillate types of spore arrangement. The substrate mycelium belonged to the yellow-brown group on ISP2 medium, indicating they might belong to the species *werraensis,* which was supported by16S rRNA gene sequence similarity.

So, the potential isolates obtained from marine sediments were identified as *Streptomyces fradiae* (KP2, TPM), *Streptomyces werraensis* MG748836 (ECR1, ECR16 and SM2) *Nocardiopsis synnemataformans* (KOM4) and *Pseudonocardia xishanensis* (PPM7).

In this investigation anti-quorum sensing activity of crude protein from isolate KP2, TPM and KOM4 using indicator organism *C. violaceum* MTCC- 2656 was also evaluated. Actinomycin D found in the marine *Streptomyces parvulus*, has been reported to possess anti-quorum sensing properties [42]. Questiomycin A and a new compound 2-hydroxyacetate-3-hydroxyacetamido-phenoxazine (HHP) from marine *Nocardiopsis dassonvillei* JS106 have been reported as anti-

quorum sensing molecule by Miao [43]. Anti-quorum sensing compound Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) was extracted from marine endophytic actinomycetes *Nocardiopsis sp.* GRG 1 (KT235640) by Ranjivgandhi [23]. Apart from these reports antibiofilm and anti-quorum sensing compound has been identified from marine actinomycetes by a number of workers [44], [45],[46],[47].

As reported by Ehiagbon are and Ogundiran [48], water quality generally affects the optimum growth of aquatic organisms [40]. ICAR [50] has also reported that poor water quality results in fish being stressed and susceptible to disease. Hence to carry out *in-vivo* studies, fifteen different physicochemical parameters were analyzed for the water in the tank before cultivating fish. The tank's water quality was found to be suitable for the cultivation of experimental fishes.

In vivo studies were performed in *L. Rohita* by artificial induction of infection through the intramuscular injection of *A. hydrophila* at a concentration of 1×10^9 CFU/ml. Earlier studies have reported 1×10^9CFUml^{-1} to be the LC_{50} dose that produces infection in fishes, resulting in 50% survival [51]. At the site of administration (1×10^9 CFUml^{-1}) of the pathogen, ulceration commenced as sloughing-off of the scales, followed by the occurrence of a hemorrhagic spot that progressed to form an epidermal lesion. It expanded and affected the internal muscles, and the fishes died by 10thD when they were left untreated.

Many treatment protocols are available for treating the infection among which swimming the fish in the treatment solution comes first. Two types of swim treatment are available. One making the fish to swim for a longer duration in a treatment tank containing low concentration of antibacterial metabolite known as immersion and another is to allow the fish to swim in a treatment solution containing higher concentration of anti-bacterial metabolite for shorter duration called as bath treatment. We adopted bath treatment and allowed the fish to swim for 1 h/ per day in a treatment tank containing10 mg/ L of antibacterial protein. Bath-treated infected fishes with crude protein showed a decrease in the diameter of the lesion by 10th D and the swelling area completely got reduced after the 5th D of treatment. Treatment of the infected fishes in isolated protein with molecular weight of 14.4KDa from *S. fradiae* TPM showed diminished lesion on 8th D itself and the swelling area got completely reduced after the 3rd D of treatment. No side effects were observed in the recovered fish after treatment with crude and low molecular weight protein.

Bath treatments of the infected fishes with commercial antibiotic (kanamycin) resulted in a decrease in the diameter of the lesion after 5th D and swelling areas completely got reduced after the 4th D of treatment. Side effects such as abnormally increase in body weight and feed intake, along with a gradual decrease in activity (swimming) and an excessive accumulation of watery fluids in the body, was observed in the recovered fishes.

Report on *in-vivo* study with the ethanol extract of *Psidium guajava* leaf showed a bacteriostatic effect on fish pathogenic bacteria [52]. Harikrishnan *et al.* [29] has also reported that extracts from medicinal plants through dip treatment were effective against septicemia infection. Platinum nanoparticles synthesized also exhibited a dose-dependent inhibition of bacterial proliferation and rescued zebra fish completely from bacterial infection [53]. So far no studies has been reported in *in-vivo* condition the effect of antibacterial protein from actinobacteria against septicemial infection of fishes.

Conclusion

From this investigation, we have identified seven potent strains belonging to *S. fradiae* (KP2, TPM), *N. synnemataformans* (KOM4), *P. xishanensis* (PPM7), and *S. werraensis* (SM2, ECR1, ECR16) from marine sediments. The crude proteins of the five isolates, KP2, TPM, KOM4, PPM7, and SM2, showed significant antibacterial activity against septicemia pathogens in *in-vitro* tests. The crude proteins of *S. fradiae* (KP2, TPM) and *N. synnemataformans* (KOM4) also showed efficient anti-quorum sensing against *C. violaceum* MTCC- 2656. *In vivo* studies revealed that the crude and purified low molecular weight protein with molecular weight of 14.4KDa from TPM was able to control the infection-induced in the major carp *labeo rohita* using the test pathogen *A. hydrophila* MTCC-1739.

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Declaration of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors Contribution

All authors contributed in the conception and design of the study, acquisition of data, analysis and interpretation of data. Drafting the article and revising it critically for important intellectual content with final approval of the submitted version was taken care by all the authors.

Data Availability Information

No data is available externally.

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