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A potent fish pathogenic bacterial killer *Streptomyces* sp. isolated from the soils of east coast region, South India

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated that ECR provides a diverse habitat for novel metabolites producing actinobacteria. The finding of antibacterial and GC-MS studies revealed that the bioactive metabolites produced by *Streptomyces* sp. ECR77 could be an effective and alternative killer against bacterial fish pathogens.
Details on Page 180

ABSTRACT

Objective: To investigate the potentiality of the marine actinobacteria isolated from marine soil against fish pathogenic bacteria.
Methods: In the present study, a total of 33 soil samples were collected from the Bay of Bengal, east coast region (ECR) of Tamilnadu, South India. Then they were used for the isolation of actinobacteria by using conventional serial dilution technique on starch casein agar medium. The antibacterial activities of the actinobacteria were screened primarily by using cross streak plate method against fish pathogenic bacteria namely *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio cholera*, *Aeromonas* sp. and *Pseudomonas* sp. The antimicrobial efficacy of the selected isolates was carried out with various organic solvents, and finally the active compound was subjected to chromatographic techniques including TLC and GC-MS.
Results: Of the 82 actinobacteria isolated, 21 (26%) isolates were possessed antibacterial activity against fish pathogenic bacteria. Out of 21 antibacterial isolates, the isolate ECR77 was selected for further study based on its potential activity against fish pathogenic bacteria. Of the various solvents tested, the ethyl acetate extract had good antibacterial activity against the tested bacterial pathogens. The isolate ECR77 grew well on oat meal agar medium with 2% salt level at 35 °C. GC-MS study found that the presence of bioactive compounds namely tetradecanoic acid, *n*-hexadecanoic acid and octadecanoic acid. The morphological, physiological, biochemical and cultural characteristics of the potential isolate were supported the identity up to generic level as *Streptomyces* sp. ECR77.
Conclusions: The results obtained from this study concludes that the ECR soils of South India is a hot spot of novel bioactive compound producing marine actinobacteria with great pharmaceutical values.

KEYWORDS

East coast region, South India, *Streptomyces* sp. ECR77, Antibacterial activity, GC-MS, Decanoic acids

1. Introduction

In recent years, fisheries sector is rapidly growing worldwide as food production system[1]. The fish diseases are mainly caused by bacteria, fungus, virus and protozoa. Particularly, bacterial diseases are responsible for heavy mortality rates in fishes. Several chemotherapeutic agents are used in the fishery sector for the treatment of fish diseases caused by bacterial pathogens. However, the fish bacterial pathogens are becoming more resistant to currently used drugs[2]. Hence, the fishermen are facing lots of challenging problems in controlling the diseases caused

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by these drug resistant bacteria, and the fishing sector is more vulnerable not only in connection with drug resistant microbial pathogens but also the pollution of marine habitats. Due to these problems, the need of hour is search/screen new effective antibacterial agents, which could be used to control the growth of fish pathogenic bacteria with environmental friendly mode.

Marine microorganisms have the ability to survive and grow in extreme environmental habitats. They have been recognized as a major source for the production of bioactive secondary metabolites. The large number and diversity of marine bacteria suggested that this resource will be of significant role in the discovery of novel drugs[3,4]. The natural compound from marine microorganisms could be the most promising bioactive agent. Among all the marine forms, the actinobacteria have special consideration in view of the proven biosynthetic capabilities of numerous isolates. Members of genus the *Streptomyces* are not only abundant in terrestrial environments but also from marine environments[5]. Approximately, 9 500 antibiotics from actinomycetes have been reported by 2008, of which 85% are from *Streptomyces* sp. Remaining are from other rare actinobacteial genera[6]. Many reports describe that the east coast region (ECR) of India is a major source of actinobacteria[7]. However, the marine streptomycetes are largely unexploited for useful metabolites. Thus, the present investigation has planned to find out the novel antibacterial agent against fish pathogen from actinobacteria isolated from the marine soils of ECR (Bay of Bengal) of Tamilnadu, India and characterize the potential antibacterial compounds and their producer.

2. Materials and methods

2.1. Isolation of actinobacteria

A total of 33 soil samples were collected from 11 different locations of ECR of Tamilnadu, South India into sterile polythene bags. The samples were serially diluted and 0.1 mL of the aliquot was spreaded over 50% sea water starch casein agar (SCA) with cyclohexamide 25 mg/mL and nalidixic acid 20 mg/mL[6] for the prevention of other microbial contaminations. All the plates were incubated at 28 °C for 7-15 d. The colonies of actinobacteria developed over the medium were purified and maintained in SCA medium.

2.2. Isolation of fish pathogens

Five fish pathogenic bacteria namely *Vibrio alginolyticus* (*V. alginolyticus*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Vibrio cholerae*(*V. cholerae*), *Aeromonas* sp. and *Pseudomonas* sp. were isolated from infected fish by spread plate technique and identified by conventional methods. The identified bacterial cultures were maintained on nutrient agar slants at 4 °C for further use.

2.3. Screening of actinobacteria for antibacterial compound production

2.3.1. Primary screening

The antibacterial activity of the actinobacteria was primarily screened by cross streak plate method against five fish pathogenic bacteria[8]. Single streak of isolated actinobacteria was streaked on one corner of SCA plate and incubated at 28 °C for 4-7 d. After obtaining a powdery ribbon-like growth, the log phased culture of bacterial cultures were streaked perpendicular to the original streak of actinobacteria and incubated at 30 °C for 24-48 h. Control plates were also maintained based on the presence and absence of inhibition zone, and the actinobacteria were selected for further study.

2.3.2. Secondary screening

The selected actinobacterial isolates were evaluated for their antibacterial efficacy by shake flask culture method[9]. The actinobacteria were inoculated into starch casein broth and incubated for 10 d in continues shaking condition (at 120 r/min) at 28 °C. After incubation, the fermented broth was filtered through filter paper (No.1 Whatman) and centrifuged (at 4 °C) at 10 000 r/min for 20 min. The filtrate was transferred aseptically into conical flasks and stored for further assay. An equal volume of organic solvents such as acetone, chloroform, ethyl acetate, petroleum ether and methanol were added separately into cell-free culture filtrate and shaken well for 2 h for the extraction of antibacterial compounds. Then, the antibacterial efficacies of the extracts were tested against bacterial fish pathogens by well diffusion method. After 24-48 h of incubation, the diameters of the inhibition zones were recorded.

2.4. Characterization of potential actinobacterium

2.4.1. Microscopy

The morphological characteristics of potential isolate were carried out according to methods recommended by the International *Streptomyces* Project (ISP)[10]. Aerial and substrate mycelia, spore chain and sporophore morphology were determined by direct light microscopic examinations of the matured colonies grown on SCA. Further, the photomicrography of the isolate was taken using phase contrast (Nikon) and scanning electron microscope.

2.4.2. Cultural characteristics

The cultural characteristics of the potential isolate was determined after incubation at 28 °C for 10-15 d on culture media recommended by the ISP[10] namely tryptone yeast extract agar (ISP1), yeast-extract malt-extract agar (ISP2), oat meal agar (ISP3), inorganic salt starch agar (ISP4), glycerol-asparagine agar (ISP5), tyrosine agar (ISP7), actinomycetes isolation agar, Kenknight agar, nutrient agar and SCA. After incubation, the growth pattern of the potential isolate such as color of the spore mass, reverse side colour and diffusible pigment production was observed.

2.4.3. Effect of temperature

The potent isolate was inoculated into SCA medium and incubated at different temperatures such as 20, 25, 30, 35, 40 and 50 °C for 10 d. After incubation, the growth was observed.

2.4.4. Effect of pH

The pH of the SCA medium was prepared with different pH such as 5, 6, 7, 8 and 9 adjusted with 0.1 mol/L NaOH/0.1 mol/L HCl. All the plates were inoculated with actinobacteria and incubated at 28 °C for 10 d and observed the growth.

2.4.5. Effect of salinity

The actinobacteria was inoculated on SCA medium with different concentration of salinity (2, 4, 6, 8 and 10%) by adding NaCl and incubated at 28 °C for 10 d. The growth of the isolate was observed.

2.4.6. Effect of carbon sources

The actinobacterial culture was inoculated onto SCA medium with different carbon sources namely glucose, maltose, mannitol, starch, glyceral and sucrose (1%) and tested the cultural characteristics of actinobacteria.

2.5. Thin layer chromatography

Thin layer chromatography (TLC) of the ethyl acetate extract of the isolate was performed to find out the antibacterial compounds on silica gel slides by using ethyl acetate:methanol:H_2O (6:4:1) as a solvent system. Chromatograms were observed under UV light and exposed to iodine vapors.

2.6. GC-MS analysis

The extracted active fraction from TLC was analyzed by gas chromatography-mass spectrometry (GC-MS). Mass spectra were recorded for each compound separated in succession by GC, the relative intensities corresponding to their retention time of the molecular ion peak and the fragmented ion peaks were normalized with respect to the base peak.

3. Results

In the present study, a total of 311 actinobacterial colonies were isolated from marine soils of ECR, Tamilnadu, India. From these 311 colonies, 82 were morphologically distinguished isolates. The actinobacterial isolates were produced powdery natured colonies with different aerial mass colour and reverse side pigments (red, brown and yellow/orange). Out of 82 morphologically different isolates, 21 (25.6%) isolates had antibacterial activity against fish pathogenic bacteria in the primary screening. Of which, 16 isolates showed activity against both *V. cholerae* and *Pseudomonas* sp., 14 against *V. parahaemolyticus*, 13 against *V. alginolyticus* and 6 against *Aeromonas* sp. Among 21 antibacterial actinobacteria, the isolate ECR77 showed remarkable antibacterial activity (4-16 mm) against all the five fish pathogenic bacteria tested in the primary screening than other actinobacteria (Table 1).

Table 1
Preliminary screening of antimicrobial activity of the actinobacteria.

Isolate code of actinobacteria	Zone of inhibition (mm)		
	V. cholarae	*V. parahaemolyticus*	*V. alginolyticus*
ECR1	4	3	–
ECR2	–	3	–
ECR4	6	–	4
ECR5	4	4	5
ECR7	7	–	8
ECR10	5	–	3
ECR11	3	4	–
ECR13	5	2	–
ECR16	–	3	5
ECR20	8	6	9
ECR23	5	–	14
ECR28	4	–	4
ECR31	6	3	–
ECR34	–	3	5
ECR64	10	3	12
ECR67	–	3	3
ECR69	4	–	–
ECR75	6	14	–
ECR77	9	4	17
ECR78	4	–	3
ECR81	–	13	–

–: No activity.

Of the 5 different solvents used for the extraction, the ethyl acetate extract showed maximum activity against *V. cholerae* [(13.66±0.47) mm] followed by *V. parahaemolyticus* [(9.66±0.94) mm], *V. alginolyticus* [(16.33±0.47) mm], *Pseudomonas* sp. [(11.33±0.47) mm] and *Aeromonas* sp. [(8.66±0.88) mm] (Table 2). The potential bioactive compound producing isolate ECR77 was produced grey coloured aerial mass

Table 2

Antibacterial efficacy of crude extracts of *Streptomyces* sp. ECR77 against fish pathogens.

Name of the Solvent	Zone of inhibition (mm)				
	V. cholerae	*V. parahaemolyticus*	*V. alginolyticus*	*Pseudomonas* sp.	*Aeromonas* sp.
Alcohol	8.66±0.88	0.33±0.47	8.33±0.44	6.33±0.47	0.00±0.00
Ethyl acetate	13.66±0.47	9.66±0.94	16.33±0.47	11.33±0.47	8.66±0.88
Petroleum ether	7.33±0.47	7.66±0.47	10.66±0.94	9.33±0.47	4.00±0.00
Methanol	6.33±0.47	6.66±0.94	4.33±0.47	8.66±0.47	4.66±0.47
Chloroform	4.33±0.47	0.00±0.00	4.66±0.47	6.33±0.47	0.33±0.47

and brownish reverse side pigment. The isolate did not produce any diffusible and melanin pigments. The isolate was positive for the biochemical characteristics such as production of citrase, cellulase, catalase and oxidase. The isolate was hydrolysed the starch and casein, and the isolate given negative results for reduction of nitrate, production of H_2S, urease production and haemolysis test. The scanning electron microscope photograph of the isolate showed that the formation of spiral spores with smooth spore surface (Figure 1).

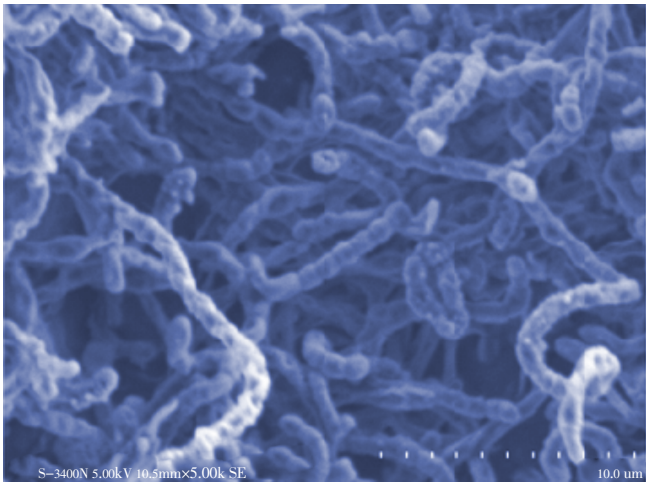

Figure 1. Scanning electron microscopic view of *Streptomyces* sp. ECR77.

The isolate grew well at temperature 35 °C, pH 8, NaCl 2-3 g/L with 1% starch (Table 3) and its growth was appeared well on oat meal agar (ISP3), actinomycetes isolation agar and SCA after 10 d of incubation. In these media, the isolate was produced grey coloured aerial mycelium and brown coloured reverse side (Table 4). The isolate also utilized all the carbon sources tested, whereas nitrogen sources like D-alanine, L-cysteine, L-isoleucine and L-arginine were not utilized by the isolate. On the basis of all the characteristics analyzed, the potential isolate has been identified to be *Streptomyces* sp. ECR77.

Table 3

Physiological characteristics of isolate *Streptomyces* sp. ECR77.

Name of the Test	Properties
Growth temperature (°C)	
20	+
25	+
30	++
35	+++
40	++
50	−
Growth pH	
5	−
6	+
7	++
8	+++
9	++
NaCl tolerance (% w/v)	
2	+++
4	++
6	+
8	−
10	−
Utilization of carbon source	
Glucose	++
Maltose	+
Mannitol	++
Starch	+++
Glyceral	+
Sucrose	+

+: Moderate; ++: Good; +++: Excellent; −: No growth.

Table 4

Cultural characteristics of *Streptomyces* sp. ECR77 on different culture media.

Name of medium	Growth	Mycelial colour		Size of the colony (mm)
		Aerial	Substrate	
Tryptone yeast extract agar (ISP1)	Good	Grey	Yellow	3
Yeast extract-Malt extract agar (ISP2)	Moderate	White	Pale yellow	2
Oat meal agar (ISP3)	Excellent	Grey	Brown	10
Inorganic salt starch agar (ISP4)	Good	Whitish grey	Colorless	5
Glycerol asparagine agar (ISP5)	Poor	White	Colorless	5
Tyrosine agar (ISP7)	Moderate	Ash	Pale yellow	2
Actinomycetes isolation agar	Excellent	Grey	Brown	9
Nutrient agar	Good	White	Yellow	7
Kenknight agar	Poor	White	Colorless	3
Starch casein agar	Excellent	Grey	Brown	8

The ethyl acetate extract of *Streptomyces* sp. ECR77 was subjected to silica gel TLC and GC-MS analyses. The TLC recorded two spots with antibacterial activity. The identity of the bioactive compounds was confirmed by the presence of peak area and molecular weight in spectral studies. These two characteristics are directly proportional to quantity of the compound present in the extract. The Figure 2 shows the GC-MS spectrum of ethyl acetate extract of *Streptomyces* sp. ECR77. Of the 22 compounds, 3 compounds had high peak percentage with retention time of 15.62 min (C1), 17.683 min (C2) and 19.527 min (C3). Based on the available library reference data, the compounds C1, C2 and C3 were determined as tetradecanoic acid (15.62 min), *n*-hexadecanoic acid (17.683 min) and octadecanoic acid (19.527 min) respectively (Figure 2).

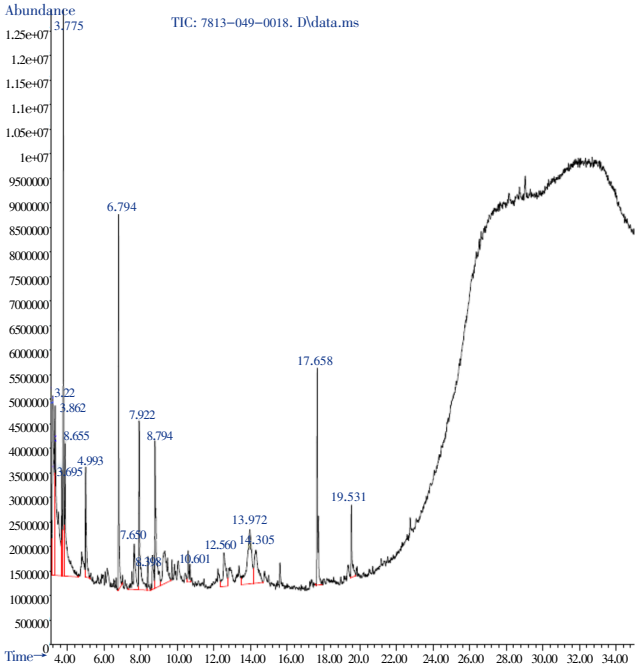

Figure 2. GC-MS analysis of bioactive compounds from *Streptomyces* sp. ECR 77.

4. Discussion

The marine soils of ECR, Tamilnadu, India were selected for the diversity and antibacterial compounds of actinobacteria. Identification of actinobacteria by various characteristics revealed that the genus *Streptomyces* showed dominant flora in ECR. The present study reported the powdery colonies produced actinobacterial isolates with different aerial mass colour and reverse side pigments (red, brown and yellow/orange). Similar type of the study has also been reported previously by Vijayakumar *et al*.[11,12]. Hence, the marine actinobacteria have worldwide distributions, which indicate their flexibility and adoptability to extremely diverse environment. In the present study, 21 isolates had antibacterial activity out of 82 isolates against fish pathogenic bacteria. From these 21 antibacterial compound producers from primary screening, the isolate *Streptomyces* sp. ECR77 was selected based on secondary screening and solubility of its antibacterial compounds in different solvents. The ethyl acetate extract of the isolate showed maximum antibacterial activity (8-16 mm) against all the fish pathogens tested. Correspondingly, Ellaiah *et al*.[13] reported that, about 25% of actinobacterial isolates had antimicrobial activity collected from marine sediments of Bay of Bengal[12]. Further, Pugazhavendan *et al*.[6] has also been reported that among different solvent extracts used, ethyl acetate extract showed strong antibacterial activity (6-15 mm) against fish pathogens[4].

The potential isolate *Streptomyces* sp. ECR77 grew well on various media such as SCA, ISP3 and actinomycetes isolation agar with pH 8.0 at 35 °C. Similarly, *Streptomyces* sp. VPTS3-2 grew well on several culture media such as SCA, ISP5 and ISP7 developed whitish and ash coloured aerial mycelium and the reverse side of the medium appeared as white, brown and ash in colour in most of the media with pH 8 at 30 °C[13]. Vijayakumar *et al*.[11]also found that the *Streptomyces* species showed effective antibacterial activity against various human pathogenic microorganisms[5]. Thus, the phenotypic properties of the isolates have been changed media to media based on the availability of substrates/nutrients.

In GC-MS spectrum of ethyl acetate extract of *Streptomyces* sp. ECR77, a total of 22 metabolic compounds were observed, which produced by the isolate. Of them, 3 compounds namely tetradecanoic acid (15.62 min), *n*-hexadecanoic acid (17.683 min) and octadecanoic acid (19.527 min) were maximally produced by the isolate. Dominant proportion of these compounds confirms that they must be responsible to the antibacterial activity. Our study has an accordance with the previous study[14], the partially purified bioactive compound was identified as silane and pyridine, 2,4,6-trimethyl, amino malonic acid and 4-benzoxazin and tris-methyl and cyclohexydimethoxy methyl compounds

Conclusively, the present study reported that ECR provide a diverse habitat for novel metabolites producing actinobacteria. The finding of antibacterial and GC-MS studies revealed that the bioactive metabolites produced by *Streptomyces* sp. ECR77 could be an effective and alternative killer against bacterial fish pathogens.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Marine microorganisms have been recognized as a major source for the production of bioactive secondary metabolites. The marine streptomycetes are largely unexploited for useful metabolites. There is a need to find out the novel antibacterial agent against fish pathogen from actinobacteria and characterize the potential antibacterial compounds and their producer.

Research frontiers

The present study characterizes the antibacterial activity of 82 actinobacteria isolated from 33 soil samples and 21 isolates were possessed antibacterial activity against fish pathogenic bacteria.

Related reports

Isolation and characterization of marine antagonistic actinomycetes from West coast of India. Isolation, characterization and antimicrobial activity of actinobacteria from Point Calimere coastal region, East coast of India.

Innovations and breakthroughs

Of the 82 actinobacteria isolated, 21 (26%) isolates had antibacterial activity against fish pathogenic bacteria. The authors have demonstrated *Streptomyces* sp. ECR77 could be an effective and alternative killer against bacterial fish pathogens.

Applications

The isolate actinobacteria of ECR77 were possessed potential activity against fish pathogenic bacteria. This scientific study supports and suggests that the ECR soils of South India is a hot spot of novel bioactive compound producing marine actinobacteria with great pharmaceutical values.

Peer review

This is a valuable research work in which authors have demonstrated that ECR provides a diverse habitat for novel metabolites producing actinobacteria. The finding of antibacterial and GC-MS studies revealed that the bioactive metabolites produced by *Streptomyces* sp. ECR77 could be an effective and alternative killer against bacterial fish pathogens.

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