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Bioactive potential of actinobacteria isolated from the gut of marine fishes

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The study was undertaken to explore the gut-associated actinobacteria from two marine fish with special reference to antimicrobial and anti-quorum sensing activity. A total of 40 actinobacterial strains were isolated from fish gut samples using starch casein agar and Kuster's agar medium. About 14 morphologically different strains recovered from *Rastrelliger kanagurta* (Indian mackerel) and *Panna microdon* (Panna croaker) were screened for the antimicrobial activity against *Staphylococcus aureus* MTCC96, *Escherichia coli* MTCC739, *Salmonella enterica*, *Candida albicans*, and quorum sensing inhibition (QSI) against *Chromobacterium violaceum* and *Serratia marcescens*. The actinobacterial strain IM20 from *R. kanagurta* showed both antimicrobial and QSI activity, whereas the strains PCA1 and PCA4 from *P. microdon* showed only antimicrobial activity. Strain IM20, which showed wide range of activity, was selected as the potential strain for further studies. Thus, the findings suggested that the fish-associated actinobacteria is a promising source for antimicrobial compounds for developing novel therapeutic drugs.

[**Keywords**: Actinobacteria; Fish gut; Antimicrobial and antiquorum sensing activity; Agar plug method]

Introduction

Research on gut-associated microorganisms of fish dates back to the early half of the 20th century[1]. Fish gut microbes play a critical role in nutrition, development, immunity, and resistant against invasive pathogens[2]. Further, recent studies revealed that fish gut-associated microbes are the promising source for secondary metabolites, enzymes, and aquaculture probiotics[3].

Actinobacteria are the group of gram positive bacteria which contain high guanine plus cytosine (G+C) in their DNA. They play a vital role in biogeochemical cycling of organic and recalcitrant materials in the environment[4]. Among the microbial resources, members of the phylum actinobacteria are well-recognized as a source for novel secondary metabolites. About two-third of the antibiotics that are commercially available in the market are produced by the members of the phylum actinobacteria, notably the genus *Streptomyces*[5]. Actinobacterial members are widely distributed in terrestrial and aquatic environments, including marine ecosystems. Even the marine sediments are the richest source for bioactive actinobacteria and the distribution of actinobacteria in marine organisms such as fishes are also documented[6,8]. However, reports on antimicrobial activity of fish gut-associated actinobacteria are very few[3]. Moreover, it has been postulated that emphasis on underexplored niches leads to the discovery of novel bioactive compounds.

The present study is an attempt for the isolation of fish gut-associated actinobacteria from two marine fish, namely, *Rastrelliger kanagurta* (Indian mackerel) and *Panna microdon* (Panna croaker), to understand their antimicrobial and quorum sensing inhibition (QSI) properties.

Materials and Methods

Sample collection and pretreatment

Marine fish, namely, *R. kanagurta* (Indian mackerel) and *P. microdon* (Panna croaker), were collected from Kovalam (Lat: 12.7870°N, Long: 80.2504°E) coastal area, Tamil Nadu. The samples were transported to the laboratory within the minimum possible time to avoid external microbial contamination. After transportation to the laboratory, the fish gut was removed[9] (Fig. 1). About 1 g of homogenates pooled intestinal segments was mixed with 100 ml of 0.85% saline in 250 ml conical flask, and the flask was kept in a shaker incubator for 30 min at 55 °C. This condition favors the isolation of actinobacteria by reducing the most unwanted Gram negative and other spore-forming bacteria[10].

Isolation of actinobacteria

Actinobacteria were isolated by adopting standard spread plate method using starch casein agar and Kuster's agar prepared with 50% sea water amended with nalicidic acid (20µg.ml^{-1}) and nystatin

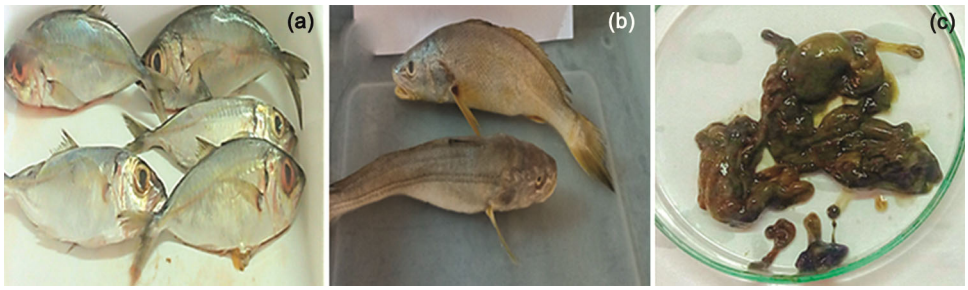

Fig. 1 — Selected marine fish: (a) *Rastrelliger kanagurta*, (b) *Panna microdon* and (c) gut portion of *Rastrelliger kanagurta*

(100µg.ml^{-1}) to inhibit the growth of both Gram negative bacteria and fungi[11]. The suspension was serially diluted up to 10^5 dilutions using sterile distilled water blank. About 100 µl of aliquot from 10^3, 10^4, 10^5 dilutions was taken and spread over agar plate using sterile L-rod. The pure actinobacteria colonies were selected and sub-cultured on yeast extract–malt extract agar medium (ISP2), and 30% glycerol and stored at −20 °C[12].

Characterization of actinobacteria

The cultural characterization was done by inoculating all the actinobacterial cultures into ISP2 agar medium. All the plates were incubated for 10 days at 28°C. The cultural characteristics that were recorded include growth, consistency, aerial mass color, the presence of reverse side pigment and soluble pigment production[13]**.** Micro-morphological characteristics were studied by adopting the slide culture method**.** About 2 ml of ISP2 agar medium inoculated with actinobacterial spores was poured as a thin layer over the surface of sterile microscopic slides. The slides were kept in sterile petri plates and incubated at 28°C for 10 days. The slides were then observed under a bright field microscope at 40x magnification. The recorded microscopic characteristics include the presence of aerial mycelium, substrate mycelium, mycelial fragmentation, and spore chain morphology.

In vitro screening of actinobacteria for antimicrobial activity

All the actinobacterial cultures were inoculated into ISP2 agar plates and incubated at 28°C for 10 days for the production of secondary metabolites. During incubation, the extracellular metabolites are secreted into the agar medium. Test pathogens used in this study include *Staphylococcus aureus* MTCC96, *Escherichia coli* MTCC739, *Salmonella enterica*, and *Candida albicans* MTCC227. The antimicrobial activity of the actinobacterial cultures was tested by adopting the agar plug method[14]. The actinobacterial cultures were grown on ISP2 agar plates and are incubated for seven days. After the incubation period, agar plug with 10 mm diameter were cut from the ISP2 agar by using well cutter and are placed in nutrient agar plate swabbed with test pathogen. All the plates were incubated at 37°C for 24 hours. The zone of inhibition was expressed in millimeter in diameter.

Screening of actinobacteria for antiquorum sensing activity

Actinobacteria were assessed for QS-inhibiting violacein production of the reporter strain *Chromobacterium violaceum* MTCC 2656 and *Serratia marcescens*. The actinobacteria isolates were cultivated on ISP2 plates for six days at 30°C. An agar plug (10mm diameter and 6mm thickness) was placed on the surface of the bioassay plates spread with overnight cultures *C. violaceum* and *S. marcescens*. The bioassay plates were incubated at 30°C for 24 hours. The appearance of turbid halo pigment less areas was assigned as QSI effect[15].

Production of bioactive compounds from the selected actinobacterial strain

The effect of medium consistency and bioactive metabolite production by the strain IM20 was investigated. Spores of the actinobacterial strain IM20 were inoculated into YEME agar plates and 100 ml of YEME broth. YEME agar plates were incubated at 28°C for 12 days. YEME broth containing flasks were incubated in rotary shaker with 95 RPM for 12 days. For every 24 hours, agar plug from YEME agar plates, and 2ml cell-free supernatant from YEME broth was taken and tested against *C. violaceum* and *S. marcescens* by adopting well-diffusion method[16].

Results

Isolation and characterization of actinobacteria

Out of 40 strains, morphologically different 14 strains were recovered in ISP2 agar slants

stored at 4 °C. During recovery and preservation, 100% of the actinobacterial cultures showed good growth on ISP2 agar. All the actinobacterial cultures showed the presence of substrate mycelium. About 86% of the cultures showed the presence of both aerial and substrate mycelium in which majority of them were *Streptomyces* as shown in Table 1.

In vitro screening of actinobacteria for antimicrobial activity

A total of 14 strains were preliminarily screened for their antimicrobial activity by agar plug method against bacterial pathogen. Strain IM20 from *R. kanagurta* showed activity against *S. aureus* MTCC96 (23 mm), *E. coli* MTCC739 (17 mm), and *S. enterica* (19 mm) (Fig. 2) (Table 2). Strain PCA4

Table 1 — Morphological characteristics of actinobacterial strains

S. No	Strain No	Cultural characteristics						
		Growth	Consistency	AMC	RSP	SP	AM	SM
1	IM1	Good	Powdery	White gray	Nil	Nil	+	+
2	IM2	Good	Powdery	White	Nil	Nil	+	+
3	IM3	Good	Powdery	White	+	Nil	+	+
4	IM18	Good	Powdery	White	Nil	Nil	+	+
5	IM20	Good	Powdery	White with light gray	Nil	Nil	+	+
6	IM22	Good	Powdery	White	Nil	Nil	+	+
7	1M23	Good	Powdery	Whitish gray	Nil	Nil	+	+
8	PCA1	Good	Powdery	Gray	Nil	Nil	+	+
9	PCA2	Good	Powdery	White	Nil	Nil	+	+
10	PCA4	Good	Powdery	Creamy white	Nil	Nil	+	+
11	PCA5	moderate	Powdery	Whitish gray	Nil	Nil	+	+
12	PCA7	Good(small)	Powdery	Gray (small)	Nil	Nil	+	+
13	PCA8	Good	Powdery	Gray(dark)	Nil	Nil	+	+
14	PCA9	Good	Powdery	Gray(light)	Nil	Nil	+	+

(+, present; Nil, absent; AMC, aerial mass color; RSP, reverse side pigment; SP, soluble pigment; AM,aerial mycelium; SM, substrate mycelium)

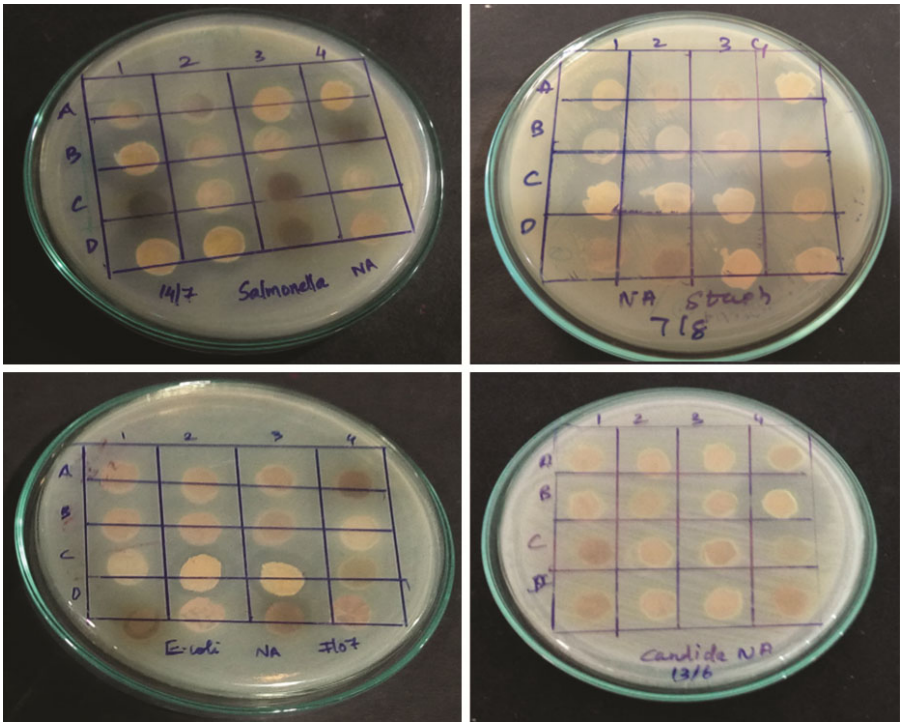

Fig. 2 — Antimicrobial activity of actinobacteria by agar plug method

Table 2 — Screening of fish gut actinobacteria for antibacterial activity

S. No	Strain	Zone of inhibition(mm in diameter)			
		S. enterica	*S. aureus*	*E.coli*	*C.albicans*
1	IM1	Nil	Nil	Nil	Nil
2	IM2	Nil	Nil	Nil	Nil
3	IM3	Nil	Nil	Nil	Nil
4	IM18	Nil	Nil	Nil	Nil
5	IM20	19	23	17	Nil
6	IM122	Nil	Nil	Nil	Nil
7	IM23	16	22	Nil	Nil
8	PCA1	15	Nil	Nil	Nil
9	PCA2	Nil	Nil	Nil	Nil
10	PCA4	19	15	20	Nil
11	PCA5	14	16	Nil	Nil
12	PCA7	Nil	Nil	Nil	Nil
13	PCA8	Nil	Nil	Nil	Nil
14	PCA9	Nil	Nil	Nil	Nil

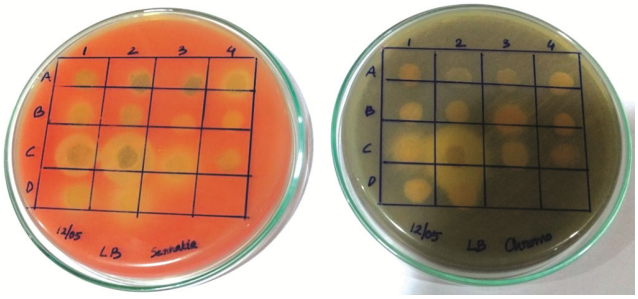

Fig. 3 — Screening of antiquorum sensing activity of actinobacterial strains by agar plug method

from *P. microdon* showed activity against *S. aureus* MTCC96 (15 mm), *E. coli MTCC739* (20 mm), and *S. enterica* (19 mm). In addition to this, the strain IM20 showed inhibition (27 mm) against *C. albicans* ATCC 90028.

In vitro screening of actinobacteria for antiquorum sensing activity

In antiquorum sensing screening activity, strain IM20 inhibited violet pigment formation of *C. violaceum* MTCC 2656 and *S. marcescens* without affecting bacterial growth (Fig. 3). From the screening, strain IM20 (Fig. 4), which inhibited all the tested pathogens, was selected for the production of bioactive substances.

Production of bioactive compounds from selected actinobacterial strain

Strain IM20 produced bioactive compounds on the third day of incubation when it was grown on ISP2 agar medium, whereas the same strain showed activity

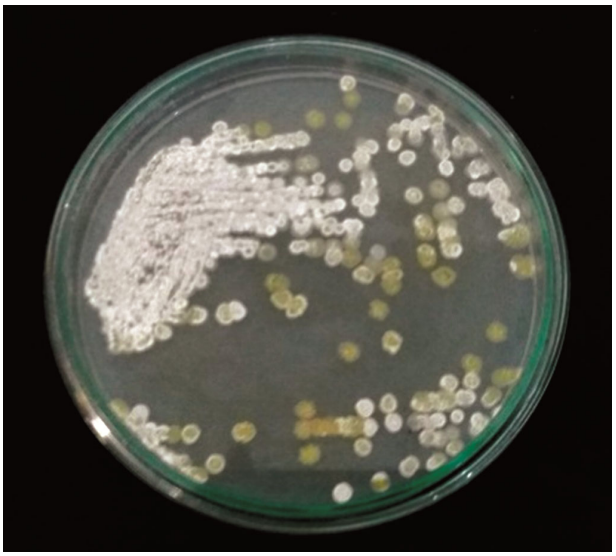

Fig. 4 — Colony morphology of potential actinobacterial strain (IM20)

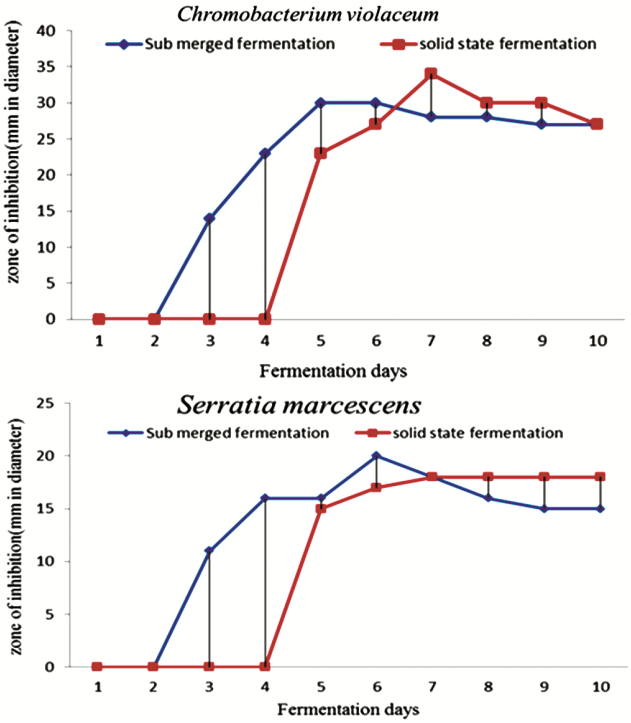

Fig. 5 — Production of bioactive compounds from selected actinobacterial strain IM20

only on the fifth day of incubation when it was grown on ISP2 broth. When compared with liquid fermentation, solid-state fermentation showed good growth and promising activity till the end of 12th day (Fig. 5). In the present study, the bioactive metabolite from strain IM20 was better produced by agar surface fermentation, a variant of solid-state fermentation.

Discussion

Historically, marine invertebrates have been a prolific source of unique natural products, with a diverse array of biological activities. Recent studies on invertebrate-associated microbial communities are revealing microorganisms as the true producers of many of these compounds. Inspired by the human microbiome project, which has highlighted the human intestine as a unique microenvironment in terms of microbial diversity, we examined the bacterial communities of fish intestines as a new source of microbial and biosynthetic diversity for natural products discovery[3].

Similar to other bacterial species, the members of actinobacteria are also reported from fish gut region. Researchers reported that about 40 actinobacterial strains that belonged to the *Streptomyces* species were isolated from the estuarine fish (*M. cephalus*, *C. chanos*, and *E. suratensis*) gills and the skin[17]. In the present study, 40 actinobacterial strains were isolated from the gut of marine fish, namely, *R. kanagurta* and *P. microdon* and screened for the antimicrobial and antiquorum sensing activity.

A very few studies on the isolation and screening of antimicrobial activity of fish-associated actinobacteria have been carried out[6,8]. But there is no previous study about the gut-associated actinobacteria for antiquorum sensing activity. In the present study, strains IM20 and PCA4 showed antibacterial activity against *S. aureus* MTCC96, *E. coli* MTCC739, and *S. enterica*. The strain IM20 showed antifungal activity against *C. albicans* ATCC90028 and antiquorum sensing activity against the violacein production of *C. violaceum* MTCC2656 and *S. marcescens* in agar plug method. The strain IM20 belonged to the *Streptomyces* species that was identified based on its phenotypic characteristics. Both the diet and the environment affect the intestinal microbiota of fish and mammals and the bacterial communities might influence the physiological characteristics of fish. In the present study, individuals of each fish harbored not similar gut bacterial communities[18]. The following two factors are responsible for such differences:

(i) Biotic factors such as nutrition and immunity,
(ii) Abiotic factors such as pH and O_2.

Therefore, improving our understanding of the dynamics of the fish gut microbiota toward dietary changes and the implications of microbial ecology modulation on bioactivity exploration will contribute to the well-being and sustainability of aquaculture.

Conclusion

The findings of the present study revealed that fish gut-associated actinobacteria are promising source for bioactive actinobacteria. *Streptomyces* species IM20 isolated from *R. kanagurta* requires further exploration with special reference to the antimicrobial and quorum sensing inhibitors.

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