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Research Article

ANTAGONISTIC POTENTIAL OF ACTINOMYCETES OF SHARAVATHI ESTUARY, KARNATAKA, INDIA

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ABSTRACT

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. The study area opted for the work was a splendid estuary in the west coast of Karnataka, Sharavathi estuary of Honnavar. 21 colonies were obtained by dilution plating method of collected samples. All the isolates were tested for antagonistic potential on 12 test pathogens by cross streak method. All the isolates were primarily screened for antibacterial activity against pathogenic bacteria, *Salmonella typhi, Proteus vulgaris, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus* species and antifungal activity against *C. albicans*, *C. neoformens, S. cerevisiae, Fusarium* and *Colletotrichum* sp. Cross streaking of test organisms perpendicular to actinomycete growth was carried out in the present investigation and antagonistic activity was determined by noting the inhibition of test organisms. *B. subtilis* was inhibited by more number of isolates followed by *S. aureus*, *P. vulgaris*, *P. aeruginosa* and *S. typhi*. *E. coli*, *K. Pneumonia* and *Streptococcus* sp were inhibited by less number of isolates. Significant inhibitory effect was observed in *C. albicans* and *S. cerevisiae* which were inhibited by more than 16 isolates. *C. lipolytica* and *C. neoformans* were inhibited by 15 isolates.

Keywords: Actinomycetes, Estuaries, Cross streak method, antagonistic

INTRODUCTION

Actinomycetes are a special group of microorganisms, which morphologically resemble Fungi and physiology of bacteria[1]. Earlier these were considered as the intermediate forms between Bacteria and Fungi. These are prokaryotic, with no nuclear envelope and membrane bound cell organelles. Hence now these are placed among bacteria. Actinomycetes are morphologically well-differentiated organisms, ranging from the simple, rapidly fragmenting, soft Nocardia to more complex forms having aerial hyphae, sclerotic granules, sporangia, or pycnidium-like fruiting bodies[2,3]. Soil is considered as the richest natural reservoir of Actinomycetes, also marine and estuarine sediments. Actinomycetes dwelling in marine habitats and estuaries are the less studied because of the difficulties in collection and isolation techniques involved as compared to soil actinomycetes[4]. An estuary is a semi-enclosed coastal body of water with one or more rivers or streams flowing into it, and with a free connection to the open sea. They are affected by both marine influences, such as tides, waves, and the influx of saline water; and riverine influences, such as flows of fresh water and sediment. As a result they may contain many biological niches within a small area, and so are associated with high biological diversity. Estuaries are typically the tidal mouths of rivers and they are often characterized by sedimentation or silt carried in from terrestrial runoff and, frequently, from offshore[5]. Estuaries provide some of the most productive habitats on earth because of the accumulation and availability of nutrients along with adequate light conditions that fuel the production of phytoplankton, the tiny, single-celled algae that drift in the water[6]. Phytoplankton is highly adapted to the nutrient-rich but often rigorous conditions of estuarine waters[7].

MATERIALS AND METHODS

Study Area

Honnavar is a taluk head quarter of Uttar Kannada district in Karnataka, India. It lies on the coast of the Arabian Sea and on the banks of the river Sharavathi, forming an estuary. It is 165 km from Shivamogga, India.

Table 1: Geographical and ecological Data of Honnavar

Latitude	$14^0 16^l$ N
Longitude	$74^0 27^l$ E
Altitude	2 meters
Average Temperature	$18\text{-}38^0$ C
Annual rainfall	200-300 cm

Figure 1: Map of Honnavar

Isolation of Actinomycetes from estuary

The station of collection was the fishing port along the estuary. The sampling was done twice, one during December and another during February 2012. During first sampling 3 samples were collected at 3 different spots ½ a kilo meter away from each other. During second sampling 2 samples was collected one km away from each other. Sterilized glass bottles of 100 ml capacity with tight rubber cork, are taken to the spot of collection. Half of the bottle was filled with sediments from 1 meter deep water. Then bottle was completely filled with surface water and the cork is resealed. These bottles were carried to laboratory, within 24 hours samples were plated.

Inoculation and Incubation

Then bottles were shaken gently and allowed to settle, the clear supernatant was then serially diluted using physiological saline up to 10^{-5} dilutions. These dilutions (1 ml of diluted suspension) were then plated on Starch Casein Nitrate, Arginine Glycerol Salt, and Kenknight and Munaier's agar media separately by pour plate method[8]. To avoid fungal contamination anti fungal such as Griesofulvin (30 mg/ltr), Chloramphenicol (30 mg) and Fluconazole (50 mg/ltr) were used in media. These plates were then incubated at 37^0C for 7-14 days. The obtained colonies were then observed and sub cultured[9,10]

Identification of Isolates

Gram's staining

Actinomycetes are gram positive bacteria with high G + C content in their genome. Gram staining was made to confirm the nature of organism.[11,12]

Cover slip method

The Actinomycetes comprise of a delicate mycelial network. Hence mounting preparations are not suitable for the morphological identification. So cover slip method was followed where SCN media was prepared and poured into the Petri plates. From solidified media agar blocks were cut and placed on the glass slide. On the agar block, isolates were point inoculated and covered by sterilized cover slip. These slides were kept in large Petri plate which was inwardly covered with blotting paper which was maintained in wet condition which acted as moist chamber. These inoculated slides were incubated at 37°C for 5 days. The moist chamber was maintained in wet condition by regular watering using sterile water. After incubation the cover slip was separated from the slide and regular mounting procedure was used for microscopic observation using crystal violet stain[13].

Antimicrobial Assay

Preliminary screening was made to check the antimicrobial activity of isolates. The method adopted was Cross streak method.

Screening of Isolates for Antibacterial Activity

Target bacteria

Gram positive bacteria: *Streptococcus* sp, *Bacillus subtilis* NCIM-2010, *Staphyloccus aureus* NCIM-2492. Gram negative bacteria: *Salmonella typhi* NCIM-2501, *Pseudomonas aeruginosa* NCIM-2200, *Proteus vulgaris* NCIM-2027, *Klebsiella pneumoniae* NCIM-2706, *Escherichia coli* NCIM-2138. The bacterial strains except *Streptococcus* sp were obtained from National Chemical Laboratory, Pune. *Streptococcus* sp was isolated from the oral cavity and identified by colony characteristics, staining reactions, biochemical characteristics and physiological characteristics. Preliminary screening of antibiotic producing strains against Gram positive and Gram negative test bacteria was tested using Cross-streak method[14]. Suspected antibiotic producing isolates were inoculated by a single streak in the centre of the Petridish and incubated at 30 ± 2°C for 3-4 days to permit growth and antibiotic production. Later the test organisms were inoculated by streaking perpendicular to the isolate streak and incubated for 24 hours at 37°C. After incubation, zone of inhibition of test bacteria around the growth of isolate was taken as criterion for primary screening[13].

Screening of Isolates for Antifungal Activity

Target fungi

Yeasts (Unicellular fungi): *Candida albicans* NCIM-3100, *Candida lipolytica* NCIM-3472, *Cryptococcus neoformans* NCIM-3541, *Saccharomyces cerevisiae* NCIM-3095. The

fungal strains were obtained from National Chemical Laboratory, Pune, India. Preliminary screening of antibiotic producing strains against test fungi was tested using Cross-streak method[14] and Point inoculation method[15]. Suspected antibiotic producing isolates were inoculated by a single streak in the centre of the petridish and incubated at room temperature for 3-4 days to permit growth and antibiotic production[16,17]. Later the test organisms were inoculated by streaking (Yeast organisms) and Point inoculation (Filamentous fungi) perpendicular to the isolate streak and incubated for 48-72 hours at 30°C. After incubation, zone of inhibition of test fungi around the growth of isolate was taken as criterion for primary screening[18,19].

RESULTS

From the estuarine sediments collected from Honnavar 21 isolates were obtained preliminarily. Out of 21 isolates, sixteen isolates belonged to the genus Streptomyces and others to Nocardia, selected for determination of antimicrobial activity.

Colony Morphology

The actinomycete colonies were found to be distinct by their cottony white, leathery, and powdery appearance on the solid media. The colour of aerial mycelium in most of the isolates was white, grey or cream with dry, cottony or powdery appearance which were later identified as *Streptomyces* isolates. Light white or yellow coloured leathery colonies observed were identified as Nocardia species. Isolates namely S3, S10, S14 and S22 were observed with characteristic coloration on the reverse side of the colony while isolates S2, S7 and S9 produced diffusible pigments (Red, Yellow and Dark green) into the medium (Table 1).

Microscopic characterization

The 21 actinomycete isolates were characterized by Cover slip method in which the typical arrangements of spores ascertain the generic specificity of actinomycetes. Most of the isolates were identified as *Streptomyces* species as noticed with straight, open loops, closed spirals and hook appearance of spores. Straight arrangement of spores was observed in case of 11 actinomycete isolates, three isolates showed open loop pattern, four isolates showed open or closed spiral arrangement.

Table 1: Colony characteristics of Actinomycete isolates

Isolate	Medium used	Pigmentation	Colony morphology	Spore formation	Spore arrangement	Tentative genera
S1	AGS	-	White, cottony with grey sporulation	+	Straight	*Streptomyces*
S2	SCA	Yellow	Creamish	+	Straight	*Streptomyces*
S3	AGS	-	Grey colored, leathery	+	Open loops	*Streptomyces*
S4	AGS	-	Ash colored, powdery appearance	+	Straight	*Streptomyces*
S5	SCA	-	Light yellow, mealy	+	Closed spirals	*Streptomyces*
S6	SCA	-	Dark grey colored	+	Flexous	*Streptomyces*
S7	SCA	Dark red	Bright white, leathery, red pigmentation	+	Primitive spirals	*Streptomyces*
S8	SCA	Red	Bright white, leathery, light red pigmentation	+	Primitive spirals	*Streptomyces*
S9	SCA	Light yellow	Creamy, tough colony, light yellow pigmentation	+	Straight	*Streptomyces*
S10	KMM	Brown	Dull white, with greenish black back	+	Short chain of spores	*Streptomyces*
S11	SCA	-	White, leathery	-	Bacilli like spores	*Nocardia*
S12	SCA	-	Creamy, mealy	+	Straight	*Streptomyces*
S13	AIA	-	White, leathery	+	Open spiral	*Streptomyces*
S14	AGS	-	Grey	+	Straight, long chain of spores	*Streptomyces*
S15	AGS	-	Creamy, yellow	+	hook	*Streptomyces*
S16	CA	-	Greyish White	-	Fragmented mycelia	*Nocardia*
S17	KMM	Brown	Dark grey, powdery	+	Short chain of spores	*Streptomyces*
S18	AIA	-	Light white powdery	+	Two open loops	*Streptomyces*
S19	CA	-	Light green, powdery	+	Long chain of spores	*Streptomyces*
S20	AIA	Brown	Light green with yellow margin	+	Closed spirals	*Streptomyces*
S21	AIA	Yellow	Light yellow; leathery	+	Long chain of spores	*Streptomyces*

Primary Screening

Results of Screening for antibacterial activity by Cross streak method are depicted in Table 2. Twenty one actinomycete isolates inhibited gram positive bacteria *B. subtilis*, 18 isolates inhibited *S. aureus* and 15 isolates *Streptococcus* sp. In case of gram negative bacteria, *P. aeruginosa* and *S. typhi* were inhibited by 16 isolates and three isolates were found to be antagonistic to *K. pneumoniae, E. coli* and *P. vulgaris* was inhibited by 17 isolates. Among bacteria tested, *B. subtilis* was found to be inhibited by more number of isolates followed by *S. aureus*, *P. vulgaris*, *P. aeruginosa* and *S. typhi*.

Table 2: Antibacterial activity of Actinomycetes in Primary screening

S. No.	Isolate No.	Antibacterial activity							
		B. subtilis	*P. aeruginosa*	*S. typhi*	*S. aureus*	*P. vulgaris*	*K. pneumoniae*	*E. coli*	*Streptococcus sp*
01	S_1	+	+	-	-	+	-	-	+
02	S_2	+	+	++	+	+	-	-	-
03	S_3	-	+	-	+	-	-	-	-
04	S_4	-	-	+	-	+	-	-	+
05	S_5	-	-	-	+	-	-	-	+
06	S_6	+	-	+	+	+	-	-	+
07	S_7	++	+++	+	+	+	+	+	-
08	S_8	+	+	+	+	+	+	+	-
09	S_9	-	-	-	+	+	-	-	-
10	S_{10}	+	+	-	-	-	-	-	-
11	S_{11}	-	+	-	-	-	-	-	-
12	S_{12}	+	-	++	++	-	-	-	-
13	S_{13}	+	+	-	-	-	-		+
14	S_{14}	+	-	-	+	-	-	-	-
15	S_{15}	+	-	+	-	+	-	-	+
16	S_{16}	+	-	+	+	+	-	-	+
17	S_{17}	+	-	+	+	-	-	-	-
18	S_{18}	+	+	-	-	+	-	-	+
19	S_{19}	+	+	-	+	+	-	-	+
20	S_{20}	+	+	+	+	+	-	-	+
21	S_{21}	+	+	+	+	+	-	-	+

Table 3 reveals results of antifungal activity of actinomycete isolates against yeasts and molds tested. It was found that *C. albicans* was inhibited by 19 isolates, *C. lipolytica* by 15 isolates, *S. cerevisiae* by 18 isolates, and *C. neoformans* by 16 isolates. Less inhibition was observed in case of *Fusarium* sp and *Colletotrichum* sp. Significant inhibitory activity was observed in case of *C. albicans* and *S. cerevisiae* followed by *C. lipolytica* and *C. neoformans.*

Table 3: Antifungal activity of Actinomycetes in Primary screening

S. No.	Isolate No.	Zone of inhibition in mm					
		C. albicans	*C. lipolytica*	*C. neoformans*	*S. cerevisiae*	*Colletotrichum* sp	*Fusarium* sp
01	S_1	+	+	+	+	-	-
02	S_2	+	+	+	+	-	-
03	S_3	+	+	+	+	-	-
04	S_4	+	+	+	+	+	+
05	S_5	+	+	+	+	-	-
06	S_6	+	+	+	+	+	+
07	S_7	+	+	+	+	-	-
08	S_8	-	-	-	-	+	+
09	S_9	+	+	+	+	+	+
10	S_{10}	-	-	-	-	-	-
11	S_{11}	+	+	+	+	-	-
12	S_{12}	+	+	+	+	-	+
13	S_{13}	-	+	-	-	-	-
14	S_{14}	-	-	-	-	-	-
15	S_{15}	-	-	-	-	-	-
16	S_{16}	+		+	+		+
17	S_{17}	-	-	-	-	-	-
18	S_{18}	+	-	-	+	+	+
19	S_{19}	+	-	-	-	+	+
20	S_{20}	+	+	+	+	+	+
21	S_{21}	+	+	+	+	+	+

DISCUSSION

Actinomycetes have been recognised as the potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunomodulators, enzyme inhibitors and many other compounds of use to man. They have provided about two third of the naturally occurring antibiotics discovered, including many of those important in medicine such as aminoglycosides, anthracyclines, chloramphenicol, β–lactams and macrolides. Many approaches like chemotaxonomical, molecular, morphological, cultural and biochemical parameters are considered in the identification of actinomycetes[7,2420]. In this study, the tentative genera of actinomycetes was assigned based on the morphological features like colour of aerial mycelium, substrate mycelium, soluble pigment produced, and characteristic spore arrangement by most convenient, simple cover slip technique. It was found that Streptomyces as the major genera followed by Nocardia. The Streptomycete isolates showed varied pattern of spore arrangements like open loops, hooks, spirals, closed spirals, straight and flexuous indicating diversity of Streptomycete isolates in estuary of Honnavar. Similar studies were also conducted by Rajkumar *et al*, 2012; Sahin *et al*. 2003; and Xiang *et al*, 1995 to identify the actinomycete populations[21-23]. Determination of bioactivity spectrum of bioactive substances from actinomycetes against

selected group of pathogens provided information on the novelty of the activity. Crude extracts of actinomycetes from from Manakkudy mangrove sediment were found to be effective on pathogenic microbes by Ravikumar et al (2011)[24]. Present investigation was successful in exploring the broad spectrum antimicrobial potential of the estuarine streptomycetes population of Honnavar. As Primary screening yielded good results, further studies on secondary screening and characterization of the antimicrobial compounds are yet to be carried out.Such screening studies were conducted by Hayakawa *et al* (2004), and Dhanashekaran *et al*. (2004) by cross streak method and agar overlay method[25,26].

CONCLUSION

The present study indicated that among the marine actinomycete isolates, *Streptomyces* is the dominant genera and revealed the diversity of marine *Streptomyces* from estuary of Honnvar and their potential as a source of novel bioactive compounds. Further studies on the molecular characterization of the isolates and purification of the bioactive compounds are in progress.

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