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Growth Inhibition of Fish Pathogens by Antagonistic Actinomycetes Isolated from Mangrove Environment

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Abstract

Solvent extracts of 6 strains actinomycetes isolated from mangrove environment were found to inhibit the growth of fish bacterial pathogens, filamentous and non-filamentous fungi. Out of the 104 actinomycetes tested 56% of the isolates exhibited antagonistic effect towards gram-negative bacteria and 35.6% towards gram-positive bacteria. The concentrations required to bring about 100% inhibition of filamentous fungi was 500-760 µg. in crude extracts. The same concentration produced inhibition zone in bacteria ranging from 2mm to 10mm in diameter against various test pathogens. Antibiogram of the actinomycetes against test pathogens showed two extreme zones (category I and Category X) of inhibition by most of the antagonistic actinomycetes. The results suggest that the crude extract of these antagonistic actinomycetes possesses both antibacterial and antifungal properties. Therefore the extract promises to be a potential source of antibiotics against fish bacterial pathogens.

Introduction

In the wake of restrictions to use antibiotics in culture operations efforts are on to search for alternate compounds which would be environment friendly. It has been observed that natural compounds from marine micro-organisms, corals and sponges could be the most promising bio-active agents. (Okazaki, and Okami (1972). In view of this the present work deals with searching of antagonistic actinomycetes for probable antibacterial, antifungal agents of mangrove origin. Mangrove environment could be a potent source for new actinomycetes harbouring biologically active substances as the mangrove sediments are rich in polymers and polysaccharides which would be a very nutritive medium for supporting microbial life [Zobell *et al.*, (1943), Wood (1953) Frietas and Bhat (1954), Weyland (1969), Walker and Colwell (1975)].

Materials and Methods

Four fixed mangrove ecosystem located along 9°55'-10°10'N and 76°10-76°20'E were selected to sample antagonistic actinomycetes.

Fortnightly collection of mangrove sediments was screened for antagonistic actinomycetes using selective medium immediately after the collection.

Actinomycetes retrieved from mangrove sediments were screened for antagonistic properties against known (fish) test pathogens provided by CIFT, Cochin and NCL, Poona. *V. anguillarum, V. cholerae, V. alginolyticus, V. parahaemaolyticus. Aeromonas, Pseudomonas, Bacillus, Staphylococcus, Salmonella I, Salmonella II, E. coli, Rhodotorula rubra, R. marina* and *Cladosporium* Sp. were the test pathogens used.

Cross-streak assay suggested by Casida (1968) was followed to study the antagonistic effect of actinomycetes.

Identification of selected isolates of actinomycetes were done with the help of the methods recommended by Shirling and Gottlieb (1966) in the International streptomycetes Project (ISP). Biochemical tests and the descriptions of the strains isolated were compared with type cultures given in ISP. Since, ISP description do not include the antagonistic property of the strains, this was compared with the available literature by Umezawa (1967) Pridham and Tresner (1974). Antimicrobial activity of the methanol extracts were tested against the test pathogens selected for the study by agar diffusion method (Casida 1968). The zone of inhibition was measured in mm excluding the diameter of cup-3mm after the incubation period (24 hours). Sea water agar was the medium used for the test, as it was found best for isolation, maintenance and also to test the antimicrobial activity in the present study.

Results and Discussion

Actinomycetes ranged from 1.0 x 10^4/gm to 243 x 10^4/gm of sediment sample. Maximum counts (243 x 10^4/gm) was recorded in the month of September 1991.

Analysis of variance of distribution of total number actinomycetes did not show any significant difference neither between the stations nor between the seasons in the distribution of total number of actinomycetes.

Among 52 cultures identified it was found that streptomycetes dominated in the mangrove sediments than that of actinomycetes. Out of 52 cultures identified, 35 (67.31%) represented the genus streptomycetes and 18 (34.62%) were of actinomycetes.

Out of 35 cultures identified as streptomycetes, maximum 13 (37.14%) were isolated from Mangalavana, followed by 11 (31.43%) from Narakkal, 8 (2.86%) from Puthuvyppu and only 3 (8.57%) from the light house area Puthuvyppu. And among 18 cultures of actinomycetes

identified, 7 were (38.89%) from Puthuvyppu, 4 (22.2%) from Narakkal and 4 from lighthouse area of Puthuvyppu and only 3 (16.67%) were from Mangalavana (Table 1).

Among 24 white colour streptomycetes isolated for identification 8 (33.33%) were from Mangalavana, another 8 (33.3%) cultures were from Narakkal, 6 (25.00%) from Puthuvyppu and 2 actinomycetes identified, 3 (21.43%) were from station1- (Table 1).

observed in Category I. *Pseudomonas* and *Aeromonas* were inhibited by 31 and 30 antagonistic actinomycetes respectively but differed greatly in their inhibitory activity. Maximum number of isolates inhibiting *Pseudomonas* were found in Category I, whereas, those inhibiting *Aeromonas* were recorded in the Category X. 33 isolates were able to inhibit Salmonella-I and the maximum inhibition was found in Category I and X. Salmonella-II was inhibited by 25 isolates and the maximum inhibition was seen in Category X. *E. coli* was the most sensitive

Table 1. Colour Pattern of 52 Identified Actinomycetes in the Study Area

Colour / Station	White	Grey	Red	Orange	Green	Total	
I	A	3	0	0	0	0	3
		(21.43%)	(0.00%)	(0.00%)	(0.00%)	(0.00%)	(16.66%)
	S	8	3	2	0	0	13
		(33.33%)	(37.50%)	(66.67%)	(0.00%)	(37.14%)	
II	A	4	0	0	0	0	4
		(28.57%)	(0.00%)	(0.00%)	(0.00%)	22.22%)	
	S	8	2	1	0	0	11
		(33.33%)	(25.00%)	(33.33%)	(0.00%)	(31.43%)	
III	A	3	0	3	0	1	7
		(21.43%)	(0.00%)	(100%)	(0.00%)	(100%)	(38.89%)
	S	6	2	0	0	0	8
		(25.00%)	(25.00%)	(0.00%)	(0.00%)	(0.00%)	(22.86%)
IV	A	4	0	0	0	0	4
		(28.57%)	(0.00%)	(0.00%)	(0.00%)	(0.00%)	(22.22%)
	S	2	1	0	0	0	3
		(8.33%)	(12.5%)	(0.00%)	(0.00%)	(0.00%)	(8.57%)
Total	A	14	0	3	0	1	18
		(77.78%)	(0.00%)	(16.67%)	(0.00%)	(5.56%)	(100%)
	S	24	8	3	0	0	35
		(68.57%)	(22.86%)	(8.57%)	0.00%)	(100%)	

9 gram-negative bacteria were used as test organism and maximum number of cultures 41 (39.4%) showed activity against *E.coli*, followed by 40 (38.5%) against *V. parahaemolyticus* and 33 (31.7%) against *V. cholerae*. 33 (31.7%) showed antagonism towards *Salmonella 1*. 21(20,2%) against *V. alginolyticus*, 31 (30.2%) against *Pseudomonas* 30 (29.8%) against *Aeromonas*, 25 (24.0%) against *Salmonella- II* and only 21 (20.2%) were able to show antagonistic effect against *V. anguillarum*.

And among the two gram-positive bacteria used as test organism, 33 (31.7%) were effective against *Staphylococcus* and 37 (35.6%) against *Bacillus* (Table 1.) Antibiogram of 104 selected actinomycetes from the selected area were done by cross streak assay.

21(20.2%) antagonistic actinomycetes showed inhibition to test pathogen *Vibrio anguillarum*. Maximum zone of inhibition was recorded in (4 6mm) Category II and X (70 80mm), (Table 2) *V. alginolytius* was also inhibited by same number of actinomycetes and maximum inhibition was found in Category X. Maximum number (40) of actinomycetes showed antagonism towards *V. parahaemolyticus* and the highest inhibition towards *V. cholerae* and the highest inhibition was

bacteria inhibited by 41 antagonistic actinomycetes and maximum inhibition was found in Category X. 37 antagonistic actinomycetes inhibited *Bacillus* and maximum inhibition zone was recorded in Category *X. Staphylococcus* was inhibited by 33 antagonistic actinomycetes and maximum inhibition zone falls in Category *X. Rhodotorula rubra* and *R. marina* were found to be inhibited by 90 isolates and maximum inhibition occurred in the zone of 70-80 mm. *Cladosporium* was inhibited by all the isolates and maximum inhibition was found in Category X. The overall picture of cross streak assay showed that only 2 extreme zones of inhibition patterns was exhibited by most of the antagonistic actinomycetes (Table 1).

Out of the 104 cultures isolated 6 cultures giving different type of inhibition (3 actinomycetes and 3 streptomycetes) were selected according to their nature of activity, (*i.e.*) active against gram-positive bacteria, gram-negative bacteria, Filamentous and non-filamentous fungi, active against gram-positive bacteria, filamentous and non-filamentous fungi, active against gram-negative bacteria, filamentous and non-filamentous fungi, active against filamentous and non-filamentous fungi and active only against filamentous fungi.

These 6 cultures were mass cultured and the extracellular

Table 2. Antagonistic property of the 104 isolates the results are grouped into 10 categories as follows

Category	Inhibition zone (mm)
I	1-3
II	4-6
III	7-9
IV	10-12
V	13-15
VI	16-18
VII	19-21
VIII	22-24
IX	25-27
X	70-80

compounds were extracted by using non-polar solvents *viz.*, Chloroform, ethyl ether and ethyl acetate at pH 4.0, 7.0 and 9.0.

Antagonistic strain 103A against test pathogens showed highest activity against *V. parahaemolyticus* at pH 4.0 and 7.0 in ethyl acetate and the original strain also showed antagonistic activity towards *V. parahaemolyticus*. *Staphylococcus* was the next actively inhibited test pathogen at pH 7.0 in ethyl acetate against *V.alginolyticus, Pseudomonas* and *E. coli*. In ethyl ether also pH 7 was found to be optimum and highest activity was found for *V.parahaemolyticus* . While using chloroform as the extraction solvent peak activity was obtained for *V. parahaemolyticus*, less intense activity was obtained for *Salmonella-I* and *Bacillus*, but original culture showed no antagonism.

Antagonistic activity of crude antibiotic extract of strain 92A against test pathogens showed highest activity against *V. parahaemolyticus* at pH 7.0 in ethyl acetate.

Strain 104 showed inhibition only towards gram-positive bacteria,i.e, *Bacillus, Staphylococcus*, non-filamentous fungi *R.marina* and towards filamentous fungi *Cladosporium*. Whereas the extract were active against gram-negative bacteria. Ethyl acetate gave the best result at pH 7 where all the test pathogens were inhibited. When extracted at pH 4.0 with ethyl acetate gave better results than at pH 7.0. whereas ethyl ether at pH 7.0 gave better results than at pH 9.0. When chloroform was used all the test pathogens were inhibited at pH 4.0 and the activity being in the zone of inhibition between 10 to 40 mm, whereas at pH 7.0 also all the test pathogens were inhibited except for *V. cholerae* and *Aeromonas*. But only *Staphylococcus* was inhibited at pH 4.0 with the chloroform extract.

Strain 187 was identified as *S. cacoi* inhibiting all the tested gram-negative bacteria, filamentous and non-filamentous fungi except the gram-positive bacteria.

Strain 202 identified as *A. mutabillis* was able to inhibit only filamentous and non-filamentous fungi, where as the crude extracts showed activity towards most of the test pathogens. Maximum activity (80mm) was shown when choloroform was used at pH 4.0 towards Salmonella-II whereas inhibition towards other test pathogens was only below 20 mm except for *Cladosporium* (above 30 mm). Ethyl ether at pH 7.0 was found to be the best which inhibited all the test organisms except *Bacillus* and *R. rubra*. And pH 9.0 showed better result than pH 4.0 where none of the test organisms were inhibited. pH 4.0 in ethyl acetate did not inhibit any of the test organisms whereas pH 7.0 showed better result where *Staphylococcus, R. marina* and *Cladosporium* were inhibited to the maximum than other test organisms. At pH 4.0 most of the test organisms were inhibited minimal inhibitory action.

Strain 190 which was identified as *A. flavascens* was able to inhibit only *Cladosporium*. The extracts were able to inhibit most of the test organisms but only with minimal inhibitory action i.e., below 20 mm. None of the antibiotic extracts showed activity more than 20 mm. Here maximum inhibition was obtained towards *V. parahaemolyticus* with ethyl acetate at pH 9.0, followed by *Bacillus, E.coli, Pseudomonas*, and *Aeromonas* and pH 4.0 was found to be the next best optimum pH of ethyl acetate, where all the test organisms were inhibited except Bacillus; pH 7.0 in ethyl acetate also inhibited *V. cholerae, Aeromonas, Pseudomonas, R. rubra, R. marina* and *Cladosporium*. In ethyl ether at pH 7.0 only *Aeromonas* and *Staphylococcus* were inhibited, where as at pH 4.0 *V. cholerae, Salmonella-II, E.coli* were inhibited and at pH 9.0, *V. parahaemolyticus, Pseudomonas* and *Bacillus* were inhibited. In all the 3 pH selected, chloroform showed activity towards most of the test pathogens but with minimum inhibition below 10 mm.

Strain 103A identified as *S. mobilis* showed the same activity in culture as well as in the extract. Ethyl acetate was found to be the best solvent at pH 4.0. Strain 190 was identified as *A. flavascens* which gave very poor antagonistic activity when extracted with ethyl ether at pH 4.0 the activity was turned to broad-spectrum inhibiting gram-positive bacteria, gram-negative-bacteria, filamentous and non-filamentous fungi.

Very few reports are available regarding the antagonistic actinomycetes from the mangrove environment. Postmaster and Freitas (1975) isolated an antibiotic producer from marshy land. Mangrove sediment form the important medium in nature for the growth, multiplication and survival of actinomycetes. actinomycetes exists in soil in complex association with environment and among them various forms of antagonistic relationships are noted. The presence of abundance of antagonistic organisms in sediment have been studied by Waksman *et al.*, (1942) and Burkholder (1946). Bioactive substances from marine actinomycetes from Sagami Bay Japan showed new and unique spectrum of antibiotic activity (Okazaki and Okami, 1972). Varieties of micro-organisms reported were more in marine sediments compared to soil samples, thus showing every possibility of getting marine bioactive substances.

Seasonal effect was found in the antagonistic activity of the actinomycetes during different seasons. Pre-monsoon recorded a high antifungal activity, especially in filamentous fungal inhibition of different test organisms, whereas antibacterial and both antibacterial and antifungal showed same intensity of inhibition towards test organisms by the antagonistic actinomycetes. Vanaja Kumar (1979) also found that pre-monsoon was the favourable season for isolating maximum number of antagonistic actinomycetes. Monsoon showed same pattern but with less intensity. Post-monsoon recorded same type of activity but the activity was still less, which may be due to 100% inhibition of the filamentous fungi.

Isolates belonging to the white colour series dominated in the antagonistic activity, showing maximum activity against gram-negative bacteria followed by gram-positive bacteria and both gram-negative and gram-positive bacteria. Grey colour series was next in order exhibiting same intensity of activity. More number of white coloured cultures exhibited combined antibacterial and antifungal activity than pigmented ones. Except orange and green all the colour series showed activity, against gram-negative bacteria. The actinomycetes from molluscs of Porto-Novo coastal region also predominated in white colour series exhibiting activity against gram-positive bacteria (14.5%). More activity exhibited by mangrove isolates against test pathogens showed the antibiotic potential of mangrove actinomycetes. This was reported by Rangaswami and Oblisami (1967) who found that there is considerable variation in the potential of actinomycete from different ecosystem. Thus mangrove proved to be a potential source of antagonistic actinomycetes, as evidenced by the present study.

Out of 104 actinomycetes 37 showed antagonism towards gram-positive bacteria, and 59 was found to be antagonistic towards gram-positive bacteria were the most susceptible group against the antagonists from soil. In the present study gram-negative bacterial inhibition was more (52.54%) whereas isolates of Porto Novo region from mollusc indicated less activity against gram-negative bacteria. White colour series dominated in gram-negative bacterial activity followed by grey, red, orange and green. Combined antibacterial and antifungal activity was exhibited by more number of grey colour actinomycetes (20.3%) than white coloured ones (19.0%) isolates with combined filamentous and non-filamentous activity were found to be the most common forms.

All the isolates exhibited antibacterial or antifungal activities. Antagonistic activity recorded were more towards gram-negative bacteria (59) when compared to both gram-positive and gram-negative bacteria, whereas, the actinomycete isolated from mollusc showed more antagonism towards gram-positive bacteria than gram-negative bacteria (Vanaja Kumar, 1979). Rangaswmi *et al.,* (1967) isolated actinomycete which was antagonistic to gram-positive and gram-negative bacteria except *E.coil* and *E. corotovora* Okazaki and Okami (1972), reported that there is a possibility of strains active against gram-positive and gram-negative bacteria from marine sediments. In marine sediments (Velankar, 1955; Matonkar, 1980) gram-negative bacteria predominated and only 5% of the total formed gram-positive forms. This may be the reason why the mangrove actinomycetes have produced antagonistic compounds inhibiting mostly gram-negative bacteria. actinomycetes isolated from mollusc produced more antagonistic compounds to inhibit the indigenous gram-positive forms. From these observation it is evident that the actinomycetes in each and every niche has a decisive factor in determining the antagonistic compounds to be produced by them.

Cladosporium, the filamentous fungi was inhibited by all the (100%) isolates. 90% of the actinomycete showed antagonism towards yeast, non-filamentous fungi. A high rate of inhibition was obtained with 90% actinomycete cultures towards filamentous and non-filamentous fungi. There were considerable variations in the nature of inhibition of different test organisms for the actinomycetes from different stations.

Antibiogram of 104 isolates from the selected area showed the nature of inhibition of test cultures. Out of 104 antagonistic actinomycetes 100% of the isolates were able to inhibit filamentous fungi. Nearly 86.5% inhibited non-filamentous fungi. Generally *Pseudomonas* and *Aeromonas* were reported to be more resistant to the existing antibiotics. Nearly 29.8% and 28.9% were able to inhibit *Pseudomonas* and *Aeromonas* respectively. 35.6% inhibited *E. coli*, 38.5% inhibited *V.parahaemolyticus* 31.7% inhibited *V.cholerae* and 31.7% inhibited Salmonella-I, 24.0% inhibited Salmonella-II. Very poor activity (20.2%) was exhibited against *V. anguillarum* and *V. alginolyticus*, which showed their virulence. In general, activity against filamentous fungi and gram-negative rods was highest followed by non-filamentous fungi and gram-positive rods.

Number of antagonistic compounds inhibiting the test pathogens *Pseudomonas* and *V. cholerae* showed maximum activity only in the minimum inhibitory zone. It may be worthwhile to mention here none of the sediment isolates from Porto Novo coastal region could inhibit *P.aeruginosa* (Lakshmanaperumalswamy, 1978) Vanaja Kumar (1979) found that 20% of the actinomycete associated with mollusc was found to produce antibiotics against *P. aeruginosa*. Out of which 16.6% showed 1-10 mm inhibition against *Pseudomonas*. The presence of actinomycetes against *Pseudomonas* in molluscs and in mangrove sediment indicated that very good prospect exist for isolation of newer antibiotics from these two sources. The presence of active principle against *Pseudomonas* in mangrove sediments and their paucity in marine sediments suggests the mangrove sediment may be potential source of newer antibiotics. Further studies in the isolated antagonistic

compounds are needed in order to produce new antibiotics effective against fish pathogens.

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