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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF ANTAGONISTIC ACTINOMYCETES FROM MANGROVE SEDIMENTS

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ABSTRACT

A total of two marine actinomycetes were isolated from different locations of the Manakudi Estuary of Arabian Sea in Tamilnadu, India. They exhibited higher antagonistic activity against the Gram positive bacteria; methicillin resistant and susceptible *Staphylococcus aureus*, *Enterobacter* sp, *Salmonella typhi, Bacillus subtilis, Klebsiella pneumoniae*, *Proteus vulgaris*. For Gram negative organism *Pseudomas auregionosa* it showed intermediate activity and no antagonistic effect towards yeast like *Candida albicans*. Pink actinomycetes (PJS) with white aerial mycelium and pink substrate mycelium and black colonies (BJS) of white aerial mycelium and yellowish white substrate mycelium showed potent inhibiting effect of other microorganisms. Biochemical analysis of PJS and BJS revealed these organisms are Gram positive, starch, mannose, glucose, sucrose, fructose, lipase and urease positive, amylase, lipase enzyme positive and urease negative. All the isolated actinomycetes were resistant to nalidixic acid, methicillin and penicillin. 16S rDNA phylogenetic typing gave ~1500 bp amplified product and it was cloned in pGEMT easy vector. Sequencing of amplified product will give the phylogeny of isolated actinomycetes and the further study on this organism may provide a new antibiotic for the welfare of human being.

INTRODUCTION

Marine environment harbors devastating harmful microorganisms (Kubanek et al., 2003) and marine flora, fauna and microorganisms are evolved to evade from the epidemics caused by the pathogens with potent secondary metabolites. Mangrove ecosystem facilitates the growth of both beneficial and harmful diverse organisms. The higher productivity in mangrove swamps supports a large number of organisms. Mangrove leaves are the foundation of the food web in coastal swamps and account for 90% of the primary productivity in such environment. Such conditions facilitate the enhanced bacterial action to break down all the organic material into dissolved nutrients (Alongi, 2002).

The diversity analysis by ribotyping with 16S rDNA phylogenetic marker showed that a group of high GC rich Gram-positive bacteria (actinomycetes) are dominant in marine sediments (Urakawa et al., 1999). The 70% of the earth's surface is covered by the oceans and it covers some of the most biodiverse ecosystems on the planet. They were not explored by the human being and they are important resource for novel actinomycetes (Mincer et al., 2002). The antibiotic-resistant nosocomial infections in hospital raise the socio-economic costs of treating hospital-acquired infections. This can be overcome either by tackle the root causes of antibiotic resistance by reducing their use and limiting current prescription practices for non-lethal human diseases or by more research to develop new antibiotics to fill the gap in the meantime. Actinomycetes play an essential role in protecting human health by having the antagonistic activity against disease causing microorganisms. Study of such kind of economically viable actinomycetes would provide an insight into the development of new antibiotics to the welfare of human health by overcoming the problems created by the multidrug resistant pathogenic microbes. Hence the present study aimed to isolate the potential antagonistic actinomycetes in marine mangrove sediments from Manakudi estuary.

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MATERIALS AND METHODS

Sample collection and isolation of actinomycets

Soil sediments were collected from the Manakudi mangrove estuary of Arabian Sea, Tamil Nadu, India with 8° 6' 12" N latitude 77° 28' 57" E longitude at 5 to 6 feet depth. The collected top 10 cm mangrove sediments were stored at 4°C until used. Soil samples were serially diluted in sterile water and spread plated over the medium containing soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO4 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4$ 20 µM, agar 15 g, seawater from mangrove habitat 1 L, and 15 µg nalidixic acid were added to inhibit the growth of other bacteria (You et al., 2005) incubated at 28°C for 3 days. The isolated actinomycetes were further grown in medium

containing glucose 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, distilled water 1 L for further analysis.

Antagonistic assay

The isolated actinomycetes were further grown on the medium with glucose 20 g, tryptone 5 g, yeast extract 5 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, distilled water 1L and assayed for their antagonistic effect against selected microorganisms. Antagonistic activity of the isolated strains BJS and PJS were performed by double-layer agar method. The actinomycetes BJS and PJS were grown on the agar plate for 32 hours and overlaid with log phase cultures of Methicillin resistant *Staphylococcus aureus* (MRSA), Methicillin susceptible *Staphylococcus aureus* (MSSA), *Enterobacter* sp., *Pseudomonas aeruginosa* (MTCC 741), *Salmonella typhi* (MTCC 733)*, Bacillus subtilis,*and *Klebsiella pneumoniae* (MTCC 109) and *Candida albicans* in 0.6 % agar nutrient medium. Plates were incubated at 37°C for 24 to 32 h.

Antibiotic susceptibility testing

Antibiotic test was carried out by Kirby - Bauer disc diffusion method (Bauer et al., 1966) with oxytetracyclin, gentamicin, rifampicin, chloramphenicol, streptomycin, penicillin, methicillin and nalidixic acid. All the media components, Mueller - Hinton agar (MHA), nutrient broth, antibiotic discs, and sterile disc dispenser were purchased from HiMedia Laboratories, India. Log phase cultures of actinomycetes strain BJS and PJS were spread on the entire surface of each MHA plate and the antibiotic discs were placed on the agar surface, incubated at 37°C for 14 h.

Biochemical and physiological characterization

The biochemical tests, urea, lipid, hydrolysis, triple sugar iron agar test, MR-VP test, catalase test, nitrate reduction test and citrate utilization test were carried out according to Cappuccino and Sherman (2004). Gelatin liquefaction, starch hydrolysis and reaction on milk were tested by following the methods of Gordon et al. (1974) and Goodfellow (1971). Cultures were incubated at 4, 25, 28, 37, 42 °C and growth properties were recorded after 3, 7, 14, 21, 28 and 35 d. the isolates were incubated in tryptic soy broth (HiMedia) and growth pattern was observed at different pH 4 to 9. Similarly the isolates were tested for the levels of tolerance to sodium chloride on nutrient agar (HiMedia) supplemented with 0 to 9 % NaCl.

Genomic DNA isolation and 16S rRNA gene amplification

Total DNA was isolated as described by Hopwood et al. (1985) with minor modifications. Mycelium grown in 25 ml NDYE was pelleted and resuspended in 5 ml Sucrose TE buffer (sucrose 300 mM, 10 mM Tris, 1 mM EDTA, pH 8.0) containing 2mg/ml lysozyme, incubated at 37°C until the complete lysis of mycelium. Then 1.2 ml of 0.5 M EDTA and 0.13% proteinase K (0.2 mg/ml) were added and mixed gently, and incubated at 30°C for 5 min. 0.7 ml of 10% SDS was added and incubated at 37 °C for 2 hr. To this mixture, 6 ml Tris- saturated phenol was added and the aqueous phase after the spin was extracted with phenol chloroform, followed by two extractions with equal volume of chloroform. To the final aqueous phase RNase was added (40 µg/ml), and incubated at 37◦C for 30 min. DNA was precipitated by cold ethanol, washed with 70% ethanol, air dried and resuspended in TE buffer. 16S ribosomal DNA (rDNA) specific universal primers FP 5'-AGAGTTTGATCCTGGCTCAG-3' and RP 5'-GGTTACCTTGTTACGACTT-3' were synthesized from Sigma Genosys, Bangalore and 16S rDNA gene was amplified 50 ng of genomic DNA. The amplified product was elecroeluted from the agarose gel and cloned in pGEMT easy vector (Promega) at *EcoR1*site according to the manufacturer's instruction. The cloned vector with the amplified product was send to the Bangalore Genei for sequencing.

RESULTS

Isolation of actinomycetes

The isolated actinomycetes in AIM (Actinomycetes Isolation Medium) were showed different morphology. After three days of incubation the colonies were appeared as chalky white spots. After five days they were showed slight difference in morphology with variation in aerial mycelial colour with white, pale pink, blue, yellow, orange, brown and black. In the subsequent sub-culturing the white, pink and black colonies were able to cultivable under *in vitro* condition. In the mother plate itself the blue, yellow, orange, brown colonies showed small pin like growth with less diameter of colony formation. Some of the black and white colonies showed the fine droplets of antibiotic exudates on their surface. In total two different isolates of actinomycetes with white, pale pink and black colonies were isolated from the mangrove sediments. All those colonies were observed under light field microscope to determine their morphological variations. They were observed under light field microscope to determine their morphological variations. Both the isolates showed different morphological characters and they were stored in glycerol stock either in spore suspension or mycelial form.

Antagonistic assay

The two different isolates were tested for their antagonistic effect towards the gram positive, gram negative bacteria and fungal microorganisms. The *in vitro* antagonistic effect was summarized in Table 1. Antagonistic test revealed that most of the isolated strains showed significant killing effect on the tested microorganisms. Of this the two actinomycete isolates exhibited higher antagonistic activity against the methicillin resistant and susceptible *Staphylococcus aureus* (MRSA and MSSA), *Enterobacter* sp, *Salmonella typhi (*MTCC 733)*, Bacillus subtilis, Klebsiella pneumoniae* (MTCC 109), *and Proteus vulgaris*.

Table 1. Antagonistic effect of isolates BJS and PJS

Microorganisms	Zone of inhibition (mm)	
	Black isolate (BJS)	Pink Isolate (PJS)
B. subtilis	35 ± 0.054	- -
S. typhi	35 ± 0.061	21 ± 0.004
Methicillin resistant *Staphylococcus aureus*	35 ± 0.023	20 ± 0.016
S.aureus	34 ± 0.024	21 ± 0.009
K. pneumoniae	23 ± 0.082	11 ± 0.051
P. aeruginosa	14 ± 0.071	11 ± 0.009
Enterobacter sp.	20 ± 0.041	19 ± 0.011
C. albicans	- -	- -

± Standard deviation

Table 2. Biochemical test of isolates BJS and PJS

Biochemical Test	Result	
	Black isolate (BJS)	Pink Isolate (PJS)
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Lipid hydrolysis	+	+
Gelatin hydrolysis	+	+
Urea hydrolysis	+	+
Catalase	+	+
Nitrate reduction	-	+
Citrate utilization	-	-
Triple sugar iron agar	+	+
Growth on MacConkey agar	-	-
Methyl Red test		
Voges-Proskaur test		
Gram's stain reaction	+	+
Cell shape	Mycelium	Mycelium
Spore formation	+	+
Motility	-	-
Growth on 0 to 7 % NaCl	Within 2 days	Within 2 days
Growth on 8 to 9 % NaCl	Within 4 days	Within 4 days
Growth on pH 5	-	-
Growth on pH 6, 7, 8, 9	+	+
Growth at 4°C	-	-
Growth at 25, 28, 37, 42°C	+	+

+ is the positive reaction and
– is the negative reaction

The gram negative bacteria *Pseudomonas aeruginosa* (MTCC 741) showed intermediate activity and no antagonistic effect towards yeast like *Candida albicans*. The Pink actinomycetes (PJS) with white aerial mycelium and pink substrate mycelium and black colonies (BJS) of white aerial mycelium and yellowish white substrate mycelium showed potent inhibiting effect of test microorganisms when compared to other isolates. Hence these two isolated were named as PJS and BJS respectively and used for further characterization.

All the test microorganisms were further tested for their susceptibility to the secondary metabolite produced by the pink actinomycete (PJS) and black actinomycete (BJS) by soft agar overlay method. The pathogenic microorganism's growth was arrested by the natural product with antibiotic effect secreted by the isolate BJS and PJS in the medium. The 32 hour culture of isolates BJS and PJS when overlaid with MRSA and MSSA, they completely arrested the growth of the above said microorganisms in the plate. The isolate BJS when overlaid with soft agar culture of *B. subtilis*, *P. aeruginosa* MTCC 741, *P. vulgaris*, *K. pneumoniae* MTCC 109 and *S. typhi* MTCC 733 exhibited the inhibition zone diameter of 12 ± 0.043, 17 ± 0.051, 14 ± 0.008, 22 ± 0.097, 33 ± 0.064 mm respectively.

Biochemical characterization

The isolates BJS and PJS were subjected to various biochemical tests and the results were depicted in table 2. The isolates BJS and PJS can able to grow on modified MG's medium supplemented with 0 to 9% NaCl. The growth of isolates was higher in media containing 0 to 7 % NaCl and they completely covered the agar surface within 2 days of incubation. In media with 8 and 9 % NaCl, they showed higher growth after 4 days of incubation.

These isolates were utilized the carbon sources; glucose, fructose, sucrose, lactose, starch, mannitol, arabinose, raffinose and xylose. The utilization of starch, tributyrin, and casein showed that these isolates produced the extra cellular enzymes amylase, lipase and protease to metabolize the polymeric components of nutrient mixture to monomeric form for the growth. Positive reaction on catalase enzyme revealed the isolates can withstand the stress condition generated by reactive oxygen species. The test on triple sugar iron agar revealed that these organisms would not produce the gas and acid when using the carbon source as glucose, sucrose and lactose. Their colony morphology with characteristic musty odour, spore formation, dimorphic mycelial forms as aerial and subterranean mycelium, gram positive non motile nature of colonies indicate that they belong to the actinomycetes group of bacterial community.

Antibiotic susceptibility testing showed the isolates were highly susceptible to the oxytetracyclin, gentamicin, rifampicin, chloramphenicol, streptomycin and resistant to penicillin, methicillin and nalidixic acid. The inhibition zone formed against these antibiotics was 19, 25, 23, 22, 28 mm for the antibiotics oxytetracyclin, gentamicin, rifampicin, chloramphenicol, streptomycin respectively.

Genomic DNA isolation and 16S rDNA gene amplification

The genomic DNA isolated from the isolates PJS and BJS were PCR amplified and the amplified product was run on 0.8 % agarose gel (Fig. 1). PCR amplification yielded approximately 1500 bp amplicon.

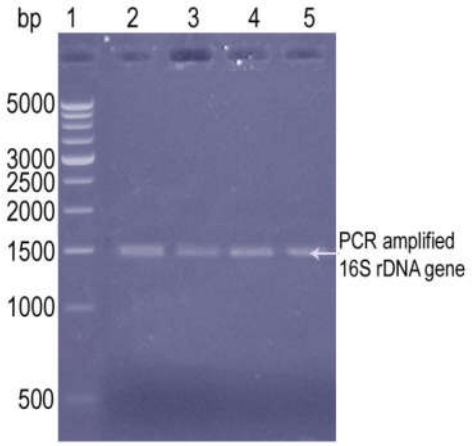

Figure 1. PCR amplified and electroeluted 16S rDNA from BJS and PJS in 0.8% agarose gel

DISCUSSION

The isolated colonies with different in morphology in terms of colour can give the conclusion that the mangrove sediment from Manakudi estuary is rich in actinomycetes diversity. Growth of some isolates like yellow, orange, brown and blue colonies were not observed in the MG's medium. After supplementing with nitrogen source in the form of peptone or yeast extract, they were not cultivable further. Hence these organisms may require the media modification with special nutrient enrichment or some physical alteration of media like pH, temperature etc for the enhanced growth and it has to be studied in detail in future.

The antagonistic activity of mangrove sediment from estuary region showed significant killing effect on the tested microorganisms. The growth of isolated actinomycetes BJS and PJS in saline condition (9% NaCl) and higher pH 9 showed that these organisms are highly salt tolerant extremophilic in nature. High degree of salt tolerance by the actinomycetes was also demonstrated by many previous studies (You et al., 2005; Okazaki and Okami, 1976) and the positive reaction of different biochemical tests for the production of extracellular enzymes and other tests revealed that the marine derived actinomycetes were metabolically active (Moran et al., 1995) and they can adapted physiologically to grow in sea water and sediments (Jensen et al., 1991).

Actinomycetes exhibit many interesting activities in water, such as degradation of many polymeric compounds into monomers such as starch and casein hydrolysis and production of antimicrobial agents (Zheng et al., 2000). The nutrient rich sediments in the estuary region provide many forms of organic supplements from plant and animal sources which will be utilized by the microorganisms in the marine both for clean up and nutrient purpose. These properties with bioactivities of the actinomycetes would play a vital role in the webs of the marine environment.

Susceptibility pattern to the antibiotic could confirm that the isolates PJS and BJS were not related to the organisms which were commercially used for the production of antibiotics like *Streptomyces rimosus*, *Micromonospora purpurea*, *Streptomyces mediterranei*, *Streptomyces venezuelae* and *Streptomyces griseus* which produces oxytetracyclin, gentamicin, rifampicin, chloramphenicol, streptomycin respectively. The detailed phylogeny of the isolates PJS and BJS can be identified after sequencing of the PCR amplified amplicon of ~1500 bp size. Detailed study of these isolates is in progress in our laboratory to know the nature and kind of antibiotic production by them.

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REFERENCES

Alongi, D.M. 2002. Present state and future of the world's mangrove forests. *Environ. Conserv*., 29, 331-349

Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *American J. Clinical. Pathol.,* 45, 493-496

Cappuccino, J.G. and Sherman, N. 2004. *Microbiology: a laboratory manual*. (Benjamin Cummings: UK)

Goodfellow, M. 1971. Numerical taxonomy of some nocardio form bacteria. *J. Gen. Microbiol*., 69, 33-80

Gordon. R.E., Barnett, D.A., Handerhan, J.E., and Pang, C.H.N.1974. *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. *Int. J. Syst. Bacteriol*., 24, 54-63.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H., Lydiate, D.J., Smith, C.P., Ward, J.M. and Schrempf, H. 1985. *Genetic Manipulation of Streptomyces: A Laboratory Manual* (John Innes Foundation: Norwich)

Jensen, P.R., Dwight, R. and Fenical, W. 1991. Distribution of actinomycetes in near-shore tropical marine sediments. *Appl. Environ. Microbiol*., 57, 1102-1108

Kubanek, J., Jensen, P.R., Keifer, P.A., Sullards, M.C., Collins, D.O. and Fenical, W. 2003. Seaweed resistance to microbial attack: a targeted chemical defense against marine fungi. *Proc. Natl. Acad. Sci., USA,* 100, 6916-6921

Mincer, T.J., Jensen, P.R., Kauffman, C.A. and Fenical, W. 2002. Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Appl. Environ. Microbiol*., 68, 5005-5011

Moran, M.A., Rutherford, L.T. and Hodson, R.E. 1995. Evidence for indigenous *Streptomyces* populations in a marine environment determined with a 16S rRNA probe. *Appl. Environ. Microbiol*., 61, 3695-3700

Okazaki, T. and Okami, Y. 1976. Studies on actinomycetes isolated from shallow sea and their antibiotic substances: in *Actinomycetes, the boundary microorganisms* (eds) T. Arai, (Tokyo: Toppan Co Ltd) pp 123-161

Urakawa, H., Kita-Tsukamoto, K. and Ohwada, K. 1999. Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiology,* 145, 3305-3315

You, J.L., Cao, L.X., Liu, G.F., Zhou, S.N., Tan, H.M. and Lin, Y.C. 2005. Isolation and characterization of actinomycetes antagonistic to pathogenic *Vibrio* spp. from nearshore marine sediments. *World J. Microbiol. Biotechnol*., 21, 679-682

Zheng, Z.H., Zeng, W., Huang, Y.J., Yang, Z.Y., Li, J., Cai, H.R. and Su, W.J. 2000. Detection of antitumor and antimicrobial activities in marine organism associated actinomycetes isolated from the Taiwan Straint, China. *FEMS Microbiol. Lett*., 188, 87-91.