Asian Journal of Chemistry Vol. 22, No. 10 (2010), 7745-7752

Screening and Isolation of Marine Actinomycetes from Visakhapatnam Sea Coast Showing Antagonistic Activity Against Selective Human Pathogenic Mirocroorganisms

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Twenty seven marine actinomycetes species were isolated from Visakhapatnam Sea Coast of Bay of Bengal and were screened for their antagonistic activity against 4 pathogenic fungal species and 7 pathogenic bacterial species. Among all the isolate No. 10 *Streptomyces rochei* (MTCC 10109) showed antagonizing effect against all pathogenic organisms studied and the antagonistic activity is very high against *Candida tropicana* and *Staphylococcus aureus*.

Key Words: Marine actinomyces, Antagonistic activity, *Streptomyces rochei*, Pathogenic fungi, Pathogenic bacteria.

INTRODUCTION

Marine actinomycetes are of considerable value as producers of antibiotics and other therapeutically useful compounds with diverse biological values[1,2]. The screenings of marine microbial natural products represent an important route for the discovery of novel chemicals and development of new therapeutic agents and also for evaluation of their potential against pathogens[3]. After the initial decades of intensive screening, it becomes difficult to isolate new actinomycetes which produce bioactive compounds[4]. Isolation of novel actinomycetes from marine environment has been a fruitful area of research for isolation of novel bioactive compounds[5].

Several antibiotics have been isolated from the marine actinomycetes by several researchers and they are unique compared to that of terrestrial sources[6,7]. However, a number of new actinomycetes strains that generate bioactive compounds have been recently isolated from novel sources, including saline, ocean and endogenic microbes of plants[8,9]. Increasing numbers of both cultured-based studies and culture-independent molecular studies show that many actinobacteria exists in marine environments such as sediments, sea water and marine invertebrates[10]. These facts

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clearly indicate that valuable microorganisms can still be isolated, from marine sources.

The Bay of Bengal has recently targeted as a potential source of marine-derived bacterial compounds by several Indian Investigators. Screening of sediment samples near the coast of the Andaman Islands in the Bay of Bengal resulted in the isolation of new marine actinomycetes and was found to be bioactive against fungi and multi drug resistant bacteria[11]. Actinobacteria produce over half of the bioactive compounds in the antibiotic literature database[12].

In the present investigation, screening and isolation of marine actinomycetes species with potent antagonistic activity against selective human pathogenic bacteria and fungi have been reported.

EXPERIMENTAL

All the chemicals used were of analytical grade and purchased from Himedia and Merck, Mumbai, India.

Collection of sea water samples: The sea water samples were collected in clean and sterilized bottles from 7 different sampling stations around Visakhapatnam Coast of Bay of Bengal and transported to the laboratory for further analysis.

Composition and pretreatment of isolation medium: The medium used for the isolation and cultivation of marine actinomycetes was starch casein medium composition: starch 1.0 %; casein 0.1 %; agar 2.0 %; 30 days aged natural sea water 50 mL and distilled water 50 mL. After autoclaving, the media was supplemented with 50 and 20 µg/mL of tetracycline and nystatin, respectively as antibacterial and antifungal agent to inhibit the bacterial and fungal contamination.

Serial dilution method: Seawater samples were serially diluted with filtered and sterilized 50 % sea water up to 10^{-2} dilutions[13]. From each suitable dilution, 0.1 mL was taken and spread evenly with sterile L-shaped glass rod over the surface of starch-casein agar (SCA) and kept for incubation at 30 °C. The actinomycetes colonies that appeared on the petri plates are counted from 7th day onwards up to 28th day and recognized by their characteristic tough, leathery appearance of colonies that adhered to the agar surface and presence of branching in vegetative mycelia[14-16].

Isolation of pure culture: Streak plate method was used to purify the marine actinomycetes colonies[17,18]. The developed colonies that grow on petri plates can be individually purified by repeated streaking on starch-casein agar medium by using separate petri plates and then subcultured to ensure for their authenticity. Pure cultures were transferred on slants and preserved at 4 °C for further analysis[7,19].

Test organisms: The human pathogenic microorganisms used for antagonistic activity were pathogenic fungi are *Candida tropicana*, *Candida albicana* (MTCC 183), *Aspergillus niger* (MTCC 1344), *Saccharomyces cercvisiae* (MTCC 307), gram positive bacteria are *Staphylococcus aureus* (MTCC 3160), *Micrococcus luteus* (MTCC 106) and gram negative bacteria are *Vibrio alginolyticus*, *Escherichia coli* (MTCC 443), *Pseudomonas aerugenosa* (MTCC 424), *Pseudomonas fluorescence*

(MTCC 103) and *Aeromonas hydrophylla*. The pathogenic microorganisms *Candida tropicana*, *Vibrio alginolyticus* and *Aeromonas hydrophylla* were obtained from Department of Biotechnology, Andhra University, Visakhapatnam, India and remaining from IMTECH, Chandigarh, India. All the cultures were maintained on nutrient slants at 4 ºC.

Screening of marine actinomycetes for antagonistic activity: Pure actinomycetes strains were grow in submerged culture in 250 mL flasks containing 50 mL of starch casein broth culture medium at 32 ºC for 7 days and centrifuged at 10,000 rpm for 15 min and the clear supernatant broth samples were tested for their antagonistic activity against the selected human pathogenic microorganisms by agar well diffusion method[20]. Wells of 6 mm diameter were prepared in the nutrient agar plates and the test pathogenic bacterial and fungal cultures were swabbed on to the nutrient agar surface[21] and the wells were filled with the 50 µL of crude culture supernatant and the diameter of inhibition zones were measured after incubation for 24 h at 37 ºC for the bacterial species and 48 h at 28 ºC in the case of fungal species.

RESULTS AND DISCUSSION

As shown in Table-1, highest number (six isolates) of marine actinomycetes isolates were observed in sampling station No. 4, an intertidal region having sandy soil and the sampling point contaminated with sewage waste disposal canal open into the sea. Whereas, sampling station No. 3 has only one isolate, which is polluted with shipyard and fishing harbour waste materials.

The degree of antimicrobial activity of the isolates was classified depending on mean diameter of inhibition zones. In the present study, the diameter of the inhibition zone divided as fallows: excellent activity (≥ 17 mm), good activity (13-16 mm) moderate activity (9-12 mm) and weak activity (≤ 8 mm).

As shown in Table-2, out of 27 isolates 3 isolates (7, 10 and 12) showed various degrees of antagonistic activity against all the 4 fungal species studied. Whereas, 5 isolates (2, 4, 11, 17, 25) showed antagonistic activity against three fungal species. The isolate No. 13 was antagonized only single fungal species.

Table-3 shows the antagonistic activity of the marine actinomycetes against pathogenic bacterial species. Out of 27 isolates, 8 isolates (1, 2, 4, 10, 11, 14, 17 and 18) were the most active against all the tested gram-positive bacteria and gram-negative bacteria. Two isolates (No. 8 and 24) were antagonized six bacterial species whereas the rest of the 4 isolates (16, 19, 20 and 25) inhibited the growth 5 bacterial species.

As shown in Table-4, *C. tropicana* was the most sensitive organism as compared to the other tested fungi, where it was inhibited by all the isolates. However, excellent degree of activity against *C. tropicana* was attained by 5 marine actinomycetes (Fig. 1).

TABLE-1
DISTRIBUTION OF MARINE ACTINOMYCETES IN DIFFERENT SAMPLING STATIONS OF VISAKHAPATNAM SEA COAST

Station No.	Sampling stations	Isolate No.	Nature of sampling stations
1	Naval dockyard sample (NDS)	1 2 3 4	Dockyard waste mixed Sea water sample
2	Ramanaidu studio sample (RSS)	5 6 7 8	Intertidal surface sea water
3	Fishing harbour sample (FHS)	9	Fishing yard waste mixed sea water sample
4	Ramakrishna beach sample (RBS)	10 11 12 13 14 15	Intertidal sea water mixed with sewage drainage canal
5	Rushi konda sample (RKS)	16 17 18 19 20	Intertidal surface sea water with low depth region
6	Tenneti park sample (TPS)	21 22 23 24 25	Intertidal surface sea water with rocky shore
7	Naval quarters sample (NQS)	26 27	Intertidal surface sea water with rocky shore

TABLE-2
ANTAGONISTIC ACTIVITY OF ACTIVE MARINE ACTINOMYCETES ISOLATES AGAINST FUNGAL PATHOGENS

Marine actinomycetes isolate No.	Tested organisms/inhibition zone (mm)			
	C. albicana	*C. tropicana*	*A. niger*	*S. cervisiae*
2	0	16	14	12
4	0	16	15	11
7	14	19	15	14
10	18	20	16	15
11	12	15	0	13
12	15	18	12	11
13	0	10	0	0
17	0	18	16	12
25	0	17	13	11

TABLE-3
ANTAGONISTIC ACTIVITY OF ACTIVE MARINE ACTINOMYCETES ISOLATES AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE PATHOGENIC BACTERIA

Marine actinomycetes isolate No.	Tested organisms/inhibition zone (mm)						
	S. aureus	*M. luteus*	*E. coli*	*V. alginolyticus*	*P. aeruginosa*	*P. fluroscence*	*A. hydrophylla*
1	13	8	10	11	10	9	12
2	18	9	11	14	12	10	10
4	16	11	14	13	11	11	14
8	8	11	9	11	0	9	12
10	19	18	15	14	12	12	15
11	10	16	8	11	10	8	8
14	9	13	12	10	10	11	8
16	13	0	11	8	0	9	8
17	17	15	13	11	11	12	11
18	14	16	12	13	10	11	10
19	15	15	10	10	0	10	0
20	12	13	0	9	0	10	8
24	11	10	0	11	8	8	8
25	9	14	8	9	0	0	11

TABLE-4
ACTIVE MARINE ACTINOMYCETES ISOLATES SHOWING DIFFERENT DEGREE OF ANTIFUNGAL ACTIVITY AGAINST THE TESTED PATHOGENIC FUNGI

Test fungi	No. of isolates/degree of activity				
	Excellent (≥ 17 mm)	Good (13-16 mm)	Moderate (9-12 mm)	Weak (≤ 8 mm)	Total No. of active isolates
C. albicana	0	4	0	0	4
C. tropicana	5	9	7	6	27
A. niger	0	6	10	1	17
S. cervisiae	0	2	17	1	19

As shown in Table-5, *Staphylococcus aureus* was the most sensitive organism among the bacterial species tested, where it was inhibited by all isolates. However excellent degree of activity against *Staphylococcus aureus* was attained by three marine actinomycetes (Fig. 2).

It was concluded that the antagonistic principles secreted by the marine actinomycetes isolates inhibited the growth of human pathogenic bacteria and fungal species studied to various degrees. Among 27 isolates, isolate No. 10 (*Streptomyces rochei*) is the most potent and exhibited excellent degree of antagonistic activity against all the pathogenic fungal and bacterial species studied and *Candida tropicana* and *Staphylococcus aureus* were the most sensitive organisms. Isolate No. 10 was deposited in Microbial Type Culture Collection and Gene Bank, Chandigarh, India and its MTCC number is 10109.

By reviewing literature[5] it was observed that aged seawater enhances the maximum production of antibiotics. Hence, in the present study for the best isolation

TABLE-5
ACTIVE MARINE ACTINOMYCETE ISOLATES SHOWING DIFFERENT DEGREE OF ANTIBACTERIAL ACTIVITY AGAINST THE TESTED PATHOGENIC GRAM POSITIVE AND NEGATIVE BACTERIA

Test bacteria	Number of isolates/degree of activity				
	Excellent (≥ 17 mm)	Good (13-16 mm)	Moderate (9-12 mm)	Weak (≤ 8 mm)	Total No. of active isolates
S. aureus	3	10	5	9	27
V. alginolyticus	0	4	18	4	26
M. luteus	0	7	6	1	14
E. coli	0	3	13	7	23
P. aeruginosa	0	0	19	2	21
P. fluorescence	0	0	20	2	22
A. hydrophylla	0	2	12	5	19

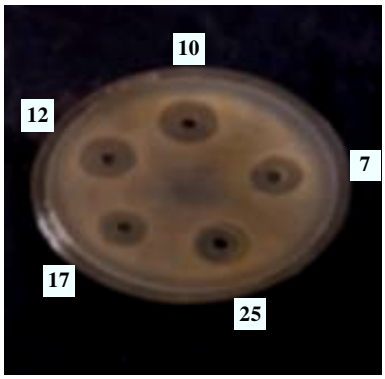

Fig. 1. Antagonastic activity of marine actinomycetes isolates (No. 7, 10, 12, 17 and 25) against *C. tropicana*

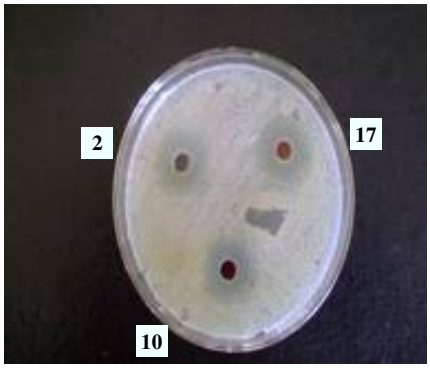

Fig. 2. Antagonastic activity of marine actinomycetes isolates (No. 2, 10 and 17 against *S. aureus*

and production of antibiotic principles by marine actinomycetes, starch-casein agar medium was supplemented with made with aged seawater[21-24].

In the present study, the isolated marine actinomycetes have exhibited excellent antifungal and antibacterial activity against *C. topicana* and *S. aureus* followed by good activity against *Aspergillus niger* and *Micrococcus luteus*, weak activity against *C. albicana* and *P. aeruginosa*. Similar results were also reported[19,25] that they observed good antibacterial activity against *S. aureus* and least activity against *P. aeruginosa* and *C. albicana* by marine actinomycetes.

Comparison of antibacterial and antifungal activities of marine actinomycetes strains have not been the focus of majority of the studies dealing with antimicrobials since most fungi require longer generation time compared to bacteria. Many antibacterial agents inhibit important steps of the formation of peptidoglycan, a very important cell wall component. On the other hand, most antifungal compounds, target either the formation or the function of ergosterol, an important component of the fungal cell membrane[26,27]. The present results also showed that most of the isolates were active against bacteria than fungi were observed the antagonistic activity by marine actinomycetes.

These results also reported that marine actinomycetes exhibited high degree of antibacterial activity against of gram positive bacteria compared to gram negative bacteria[28,29]. The reason for differential sensitivity between gram positive and gram negative bacteria could be described to the cell wall composition between these microorganisms, gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes; the gram positive bacteria are susceptible because they are having only an outer peptidoglycan layer which is not an effective permeability barrier[30].

ACKNOWLEDGEMENT

The authors are grateful to GITAM University management for providing facilities required to carry out this work.

REFERENCES

1. V.S. Berman, M. Greenstein and W.M. Maiese, *Adv. Appl. Microbiol.*, **43**, 7 (1997).
2. S.L. Sharma and A. Pant, *Indian J. Mar. Sci*., **30**, 146 (2001).
3. D.J. Kurtboke and H.G. Wildman, *Actinomycetes*, **9**, 1 (1998).
4. W. Ferical, *Trends Biotech*., **15**, 339 (1997).
5. M. Saha, D. Ghose Jr., D. Ghose, D. Garai, P. Jai Sankar, K.K. Sarkar, P.K. Dutta, S. Das, T. Jha and J. Mukarjee, *Appl. Mcirobial. Biotechn*., **66**, 497 (2005).
6. Y. Okami and T. Okazaki, *J. Antibio*., **25**, 456 (1972).
7. P. Ellaih and A.P.C. Reddy, *Indian J. Mar. Sci*., **16**, 134 (1987).
8. V.J. Mukku, M. Speitling, H. Laatsch and E. Helmice, *J. Nat. Prod*., **63**, 1567 (2000).
9. Y.G. Chen, X.L. Jiane, W.J. Cui Li, S.K. Tan, M.L. Wen, C.L. Jiahng and L.H. Xu, *J. Yunnan Univ*., **28**, 444 (2006) in Chinese.
10. T.J. Mincer, P.R. Jensen, C.A. Kaufmann and W. Dernical, *Appl. Environ. Microbiol*., **68**, 5005 (2002).
11. S. Peela and V.V.S.N.B. Kurada and R. Terli, *World J. Microbial. Biotech.*, **21**, 583 (2005).
12. A. Lazzarini, L. Cavaletti, G. Toppo and F. Marinelli, *Antonoe von leeuwenhoek*, **78**, 399 (2000).

13. M.K. Sahu, K.S. Kumar and L. Kanan, *Proc. Ecotech*., **24**, 45 (2004).
14. D.H. Bergy, Manual of determinative bacteriology, Actinomycetales, edn. 9 (2000).
15. M. Takizawa, R.R. Colwell and R.T. Hill, *Appl. Environ. Microbiol*., **59**, 997 (1993).
16. T.J. Mincer, W. Fenical and P.R. Jensen, *Appl. Environ. Microbiol*., **71**, 7019 (2005).
17. S.T. Williams and T. Cross, *Methods Microbiol*., **4**, 295 (1971).
18. D. Singh and V.P. Agarwal, Microbial Biodiversity of Mount Everest Region, a Paper Presented in International Seminar on Mountains-Kathmandu, (Organized by Royal Nepal Academy of Science and Technology), March 6-8 (2002).
19. C.R. Kokare, K.R. Mahadik and S.S. Kadam and C.R. Chopade, *Indian J. Mar. Sci*., **33**, 248 (2004).
20. I. Saadoun and A. Muhana, *Curr. Trends Biotech. Pharm*., **2**, 402 (2008).
21. A. Mitra, S.C. Santra and J.D. Mukharjee, *Appl. Microbiol. Biotech.*, **80**, 685 (2008).
22. E. Kuster and S.T. Williams, *Nature*, **202**, 928 (1964).
23. H.M. Rifaat, *J. Mediterranean Ecol*., **4**, 5 (2003).
24. R.R. Ratnakala and V. Chandrika, *Indian J. Mar. Sci*., **22**, 297 (1993).
25. K. Kathresan, R. Balagurunathan and M. Selvam, *Indian J. Biotech*., **4**, 271 (2005).
26. M.A. Ghannoum and L.B. Rice, *Clin. Microbiol. Rev*., **12**, 501 (1999).
27. D. Nedialkova and M. Naidenova, *J. Culture Collect.*, **4**, 29 (2004).
28. B. Pandey, P. Ghimire and V.P. Agarwal, Studies on the Antibacterial Activity of Actinomycetes Isolated the Khumbu Region of Mt. Everest. A Paper Presented by the International Conference on the Great Himalayan: Climate, Health, Ecology, Management and Conservation, Kathmandu University and the Aquatic Ecosystem Health and Management Society, Canada (2004).
29. M. Oskay and A.U. Tamer and C. Azeri, *Afr. J. Biotech*., **3**, 441 (2004).
30. R. Scherrer and P. Gerhardt, *J. Bacteriol*., **107**, 718 (1971).

(*Received*: 8 February 2010; *Accepted*: 26 July 2010) AJC-8897