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Antagonistic Activity of the Barnacle (*Balanus amphitrite*) Associated Bacteria Against Human Bacterial Pathogens

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Abstract: Bacterial strains associated with the barnacle *Balanus amphitrite,* distributed along Tuticorin coast, were studied for antagonistic activity against 10 human pathogenic bacterial strains. In cross streaking antibacterial assay involving the strains as such, antagonistic activity was observed in 14.3% of the 28 isolated associated bacteria. Out of four strains which showed antagonistic activity, the strain B3 showed higher antagonistic activity against 90% of bacterial pathogens. The subsequent culture of four active strains and screening of supernatant diethyl ether, chloroform, ethyl acetate and butanol extracts showed wide spectrum activity in ethyl acetate extract of B3 strain. The partitioning and column fractionation of supernatant ethyl acetate extract of B3 strain indicated wide spectrum antibacterial activity indicating the non-polar nature of the active substance. The strain B3 produced exocelluar metabolites, which though may have an ecological role to play with in nature, may provide a vital lead to antibacterials to combat human bacterial pathogens.

Key words: Antagonistic activity · Barnacle *balanus amphitrite* · Human pathogens

INTRODUCTION

The world's oceans, which cover more than 70% of the earth's surface, has been considered as a rich source of compounds possessing novel structures with rich biological activity. The iIncreasing incidence of antibiotic resistance among bacterial pathogens and emerging new diseases are posing great challenges to humans. Given the widespread misuse and over prescription of antibiotics by the medical community, the antibiotics available today are rapidly becoming less and less effective in the face of emerging multi-drug resistant pathogens of clinical concern.

The marine resources, especially the bacteria possess novel metabolites of biomedical importance. Though many potential compounds have been isolated from other organisms, continuous production poses a great challenge. In this context, the presence of potential bioactive substances in marine bacteria especially surface associated bacteria is promising, considering the requirement of new class of antibiotics to combat drug resistance and new diseases.

Microorganisms are not only the cause of infections; they can also produce organic substances that can cure infections [1]. The first marine bacterium based antibiotic was characterized in 1966 [2]. Marine microorganisms have become an important source in the search for novel microbial metabolites. Many microorganisms contain substances that have antimicrobial, antiviral, anticoagulant and cardio active properties. A few of these substances have unique chemical structures that are unlike any other compounds, may serve as leads to the discovery of new drugs. The *Streptomyces* sp. isolated from the surface of a jellyfish produced two bicyclic peptides, salinamides A and B [3]. Likewise, *Bacillus* isolated from a marine worm produced a novel cyclic decapeptide antibiotic lolatin B [4]. The bacterium *Pelagiobacter variabilis* isolated from the seaweed *Pocockiella variegata* produced phenazine antibiotics, pelagiomicins [5]. A microbial metabolite with anti-HIV potential (as reverse transcriptase inhibitor) has been developed from marine *Alteromonas* spp. isolated from the tissue of Bermudian marine sponge [6]. Some *Vibrio* species have been found to produce a variety of extra cellular proteases. The production of antimicrobial compounds by marine bacteria is usually assayed under straight forward growth conditions and only strains, which constitutively produce such compounds, can be successfully screened. However, as the primary role of antimicrobial activity can be to antagonize competitors, bacteria may also produce antimicrobial compounds when they sense the presence of competing organisms [7].

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In the present study, the community of marine bacteria associated with the barnacle *Balanus amphitrite* was considered to explore the potential lead for novel antibiotic substances.

MATERIALS AND METHODS

Isolation of Associated Bacteria: The associated bacteria from the barnacle *Balanus amphitrite* (Arthropoda: Maxillopoda: Sessilia: Balanidae), collected and transported to the laboratory in sterile plastic bags from Tuticorin Coast (Lat. 8° 45' N, Long. 78° 10'E), was isolated [8, 9]. The shells were gently broken and the sample surfaces were gently washed with sterile seawater, so that only bacteria with a strong affinity for the host were sampled. The surface was swabbed with a sterile cotton swab. Swabs were then transferred to the test tubes containing sterile seawater and mixed for 30 seconds. The swabs were removed from the tubes and the resulting bacterial suspensions were vigorously hand shaken. Serial dilutions of each solution were prepared and aliquots (1ml) were plated on Zobell Marine Agar (ZMA) (Himedia, Mumbai) plates in replicates. The plates were incubated for 48 hrs at room temperature. Colony forming units were counted and the average number of colonies was expressed as CFU/cm^2 Triplicate sampling was carried out and the average was taken. Selected colonies were restreaked onto Zobell Marine Agar (ZMA) to have pure cultures. Bacterial isolates were kept on ZMA slants at 4°C for further studies.

Antibacterial Assay: The barnacle associated bacteria were tested for antagonistic activity against 10 human pathogenic bacteria through antibacterial assay. The cross streaking method [10] was used for antibacterial assay with modification of expression of the results in percentage of inhibition.

The associated bacterial strains were individually applied as a single streak on the Zobell Marine agar (Himedia, Mumbai) plate and incubated at 28°C for 48 hours. Then the 10 test human bacterial strains (*Salmonella paratyphi, Klebsiella pneumoniae, Vibrio cholerae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Staphylococcus epidermidis, Escherichia coli, Bacillus cereus, B. subtilis* and *Enterobacter aerogenes*), obtained from Christian Medical College (CMC), Vellore, were applied as single streaks perpendicular to the associated bacterial streak without touching it [10]. Simultaneously, individual control plates without associated bacterial streak were also maintained. The plates were incubated for another 24 hours and the inhibition zones were compared and scored with that of control as high (81-100%, inhibition), Moderate (21-80% inhibition), low (1-20% inhibition) and nil (no activity).

Antibacterial Activity of Associated Bacterial Strain Extracts: The producer strains B1, B2, B3 and B4 were broth cultured in 60 ml Zobell marine broth for 5 days at 280 rpm in room temperature. The culture broth was then centrifuged at 5000 rpm for five minutes and the supernatant was collected. Equal volume of diethyl ether was added to the supernatant, mixed well for 30 minutes using a magnetic stirrer and transferred to a separating funnel. The diethyl ether phase was removed and concentrated. The supernatant was sequentially extracted using chloroform, ethyl acetate and butanol, collected separately and evaporated. The concentrated supernatants were screened against 10 human pathogens for antibacterial activity.

The 10 human pathogenic bacterial strains were inoculated in Muller Hinton Agar and incubated for 24 hours before being used in the antibacterial assay. Then the culture plates were seeded with the individual human pathogenic strains. Standard disc diffusion method [11, 12] was followed for the antibacterial assay. The sterile Whatman No 1 filter paper discs of 6 mm diameter were impregnated with 50 µl/disc of the individual extract, air dried and placed on the marine agar plates and incubated for 18-24 hours at 30°C. The assay was carried out in triplicate. Zone of inhibition was measured from the edge of the disc to the clear zone in millimeter.

Partitioning of Supernatant Extract: The partitioning of the supernatant extract was carried out to assess the polarity and to localize the active component. The crude ethyl acetate supernatant extract of strain B3 was partitioned between ethyl acetate and water and then this water phase was subsequently partitioned against butanol [13-15]. The partitioned extracts (Ethyl acetate, butanol and water), collected separately, evaporated and concentrated, were screened for antibacterial activity against human pathogenic bacteria.

Column Fractionation: The ethyl acetate extract of B3 strain was partially purified through column fractionation [14] using normal phase silica gel column chromatography employing a step gradient solvent system from low to high polarity, viz. Hexane 100%, Hexane 75%: Ethyl acetate 25%, Hexane 50%: Ethyl acetate 50%, Hexane 25%: Ethyl acetate 75%, Ethyl acetate 100%, Ethyl acetate 75%: Methanol 25%, Ethyl acetate 50%: Methanol 50%, Ethyl acetate 25%: Methanol 75%, Methanol 100%.

RESULTS

Associated Bacteria from Barnacle: The associated bacterial density in *Balanus amphitrite* was found to be 41 x 10^{-1} CFU/cm^2. Twenty eight associated bacteria were isolated based on colony morphology. The bacteria were not identified and were given designated codes.

Antibacterial Activity of Associated Bacterial Strains: Out of 28 surface associated bacteria screened, four strains (B1, B2, B3 and B4) (14.3%) showed antagonistic activity (Table 1). The other associated bacteria did not show activity and hence, the results were not presented.

Table 1: Antibacterial activity of balanus associated bacterial strains

	Inhibition level (%)			
Human pathogens	B1	B2	B3	B4
Salmonella paratyphi	Nil	Nil	30	Nil
Klebsiella pneumoniae	Nil	14	67	25
Vibrio cholerae	Nil	Nil	Nil	Nil
Pseudomonas aeruginosa	17	8	85	Nil
Streptococcus pneumoniae	12	Nil	17	17
Staphylococcus epidermidis	Nil	20	89	Nil
Escherichia coli	20	17	90	27
Bacillus cereus	8	17	20	45
Bacillus subtilis	17	Nil	43	40
Enterobacter aerogenes	Nil	40	47	15

High activity (81-100%, inhibition), Moderate (21-80% inhibition), low (1-20% inhibition) and Nil (no activity)

Table 2: Antibacterial activity of supernatant crude extracts of associated B3 bacterial strain

	Zone of inhibition (mm)			
Human pathogens	DEE	C	EA	B
Salmonella paratyphi	-	-	2	-
Klebsiella pneumoniae	-	-	4	-
Vibrio cholerae	-	-	5	-
Pseudomonas aeroginosa	-	-	1	-
Streptococcus pneumoniae	-	-	6	-
Staphylococcus epidermidis	-	-	1	-
Escherichia coli	-	-	5	-
Bacillus cereus	-	-	2	-
Bacillus subtilis	-	-	2	-
Enterobacter aerogenes	-	-	2	-

(DEE-Diethyl ether, C- Chloroform, EA-Ethyl Acetate, B- Butanol)

The associated bacterial strain B3 showed higher activity against three pathogens and moderate activity against six pathogenic bacteria. Epibacterial strains B1 and B2 showed low activity against five and six bacteria respectively and B4 strain showed moderate activity against four pathogenic bacteria and low activity against two bacteria.

Antibacterial Activity of Supernatant Extract: Among the strains B1, B2, B3 and B4, only the B3 strain showed activity (results shown only for strain B3-Table 2). The B3 bacterial strain showed broad spectral activity against all pathogenic bacteria in ethyl acetate extract. The zone of inhibition ranged from 1 to 6 mm. The highest activity was observed against *Streptococcus pneumoniae* (6 mm). No activity was observed for other extracts.

Antibacterial Activity of Partitioned Extract: The partitioning and subsequent screening of the B3 ethyl acetate extract showed wide spectrum inhibitory activity in ethyl acetate phase (Table 3). No activity was observed in butanol and water phases. The extract showed 5 mm zone of inhibition for *Bacillus cereus* and 4 mm zone for *Vibrio cholerae and Streptococcus pneumoniae*.

Table 3: Antibacterial activity of partitioned extract of B3 bacterial strain

	Zone of inhibition (mm)		
Human pathogens	EA	B	W
Salmonella paratyphi	2	-	-
Klebsiella pneumoniae	3	-	-
Vibrio cholerae	4	-	-
Pseudomonas aeruginosa	1	-	-
Streptococcus pneumoniae	4	-	-
Staphylococcus epidermidis	2	-	-
Escherichia coli	1	-	-
Bacillus cereus	5	-	-
Bacillus subtilis	2	-	-
Enterobacter aerogenes	3	-	-

(EA-Ethyl Acetate, B- Butanol, W-Water)

Table 4: Antibacterial activity of column fractions of B3 strain supernatant

	Zone of inhibition (mm)								
Human Pathogen	H100%	H 75%: EA 25%	H50%: EA50%	H25%: EA75%	EA100%	EA75%: M25%	EA50%: M50%	EA25%: M75%	M100%
Salmonella paratyphi	Nil	Nil	Nil	3	2	Nil	Nil	Nil	Nil
Klebsiella pneumoniae	Nil	Nil	Nil	5	2	Nil	Nil	Nil	Nil
Vibrio cholerae	Nil	Nil	Nil	4	1	Nil	Nil	Nil	Nil
Pseudomonas aeruginosa	Nil	Nil	Nil	6	1	Nil	Nil	Nil	Nil
Streptococcus pneumoniae	Nil	Nil	Nil	4	1	Nil	Nil	Nil	Nil
Staphylococcus epidermidis	Nil	Nil	Nil	3	Nil	Nil	Nil	Nil	Nil
Escherichia coli	Nil	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
Bacillus cereus	Nil	Nil	Nil	2	Nil	Nil	Nil	Nil	Nil
Bacillus subtilis	Nil	Nil	Nil	4	Nil	Nil	Nil	Nil	Nil
Enterobacter aerogenes	Nil	Nil	Nil	3	Nil	Nil	Nil	Nil	Nil

Antibacterial Activity of Column Purified Fractions: The chromatographic fraction of the B3 strain exhibited broad spectral activity against human pathogens (Table 4). The highest inhibition was found in elution fractions (Hexane: Ethyl acetate) 25:75%. The 100% of ethyl acetate fraction showed 50% of activity. The highest inhibition zone was recorded against in *Pseudomonas aeruginosa* (6 mm) and *Klebsiella pneumoniae* (5 mm).

DISCUSSION

The marine environment harbours a wide range of microbes capable of exhibiting bacteriolytic and antibiotic activity and the primary role of the antibiotic substances could be attributed to ecological competition. The beneficial associations between associated bacteria and their hosts have been widely reported. The associated bacteria, due to their ecological significance and evolution, produce novel chemical substances and hence, may form the basis of new drug leads. In recent years, the marine microorganisms were shown to produce molecules with novel structures and biological activities [16, 17]. The associated bacteria on the larvae of some crustaceans produce antimicrobial compounds that protect the host from fungal infection [18]. The associated bacteria isolated from a tunicate inhibited the settlement of barnacle and tunicate larvae [19].

In spite of the fact that many organisms, especially sedentary forms, produce secondary metabolites to control epibiosis of their surface; most of them harbour microbes, indicating their selective inhibitory characteristics. Studies have shown that high proportions of marine epibiotic bacteria secrete secondary metabolites with antibacterial properties [7, 20].

In the present study, the surface associated bacterial density in barnacle *Balanus amphitrite* was less when compared to the surrounding seawater. The postulation that certain surface characteristics of plants and animals play key role in controlling epibacterial population density on their surface [21, 22] coincides with the present observation. Also, the associated bacteria may have the potential to influence the composition of the microbial community present on organism surfaces through production of antimicrobial metabolites and repellents [23]. The low density was further substantiated by the observation that the biofilm bacteria on the surfaces of marine organisms contain a higher proportion of antibiotic producing strains [24] as revealed by the antagonistic activity observed in the four barnacle associated bacteria against pathogens, which coincided with earlier observations [25, 26]. The activity in barnacle associated bacteria was substantiated by the reported observation of antibacterial activity in five of the epiphytic strains against human pathogenic bacteria *Enterobacter faecalis*, *S. aureus* etc. [27].

The fact that 14.3% of the associated bacterial strains from the barnacle showed antibacterial activity was comparable to that of 8.8% out of the 45 epibiotic bacteria isolated from different marine samples, which displayed anti-*Staphylococcus* activity [28] and 12% of 341 strains isolated from seawater, sediment and marine macroorganisms from different costal areas of China [29].

The discovery of new classes of antibiotics is necessary due to the increased incidence of multiple resistances among human pathogenic microorganisms to conventional drugs [30]. Among the tested associated bacterial strains of *Balanus amphitrite*, only B3 exhibited maximal activity against all the ten human bacterial pathogens. This has implied that this particular strain may play a role in modulating the associated bacteria number in barnacles by producing antagonistic metabolites. The compositions of bacterial exopolymers, which act as chemical cues, have been shown to influence the subsequent settlement by invertebrate larvae [31, 32]. So, the bacteria associated with barnacle may play a similar role and the antibacterial activity could be explained to the production of exopolymers as the activity in supernatant has been observed in the present study.

The observed zone of inhibition was less when compared to gorgonids associated bacteria, which showed an inhibition zone up to 22 mm [33]. But, the results are comparable to the antibiotic activity of the epiphytic marine bacteria from intertidal seaweeds with a zone of inhibition of greater than 10 mm against *Staphylococcus aureus*, *Escherichia coli*, *Alcaligens feacalis* etc. [24]. The present activity was higher when compared to the moderate activity (1-5 mm) in the ethyl acetate extract of two sponge associated bacteria against pathogenic bacteria [34].

The activity in partitioned ethyl acetate phase of the strain B3 supernatant extract and that of column fraction Hexane: Ethyl acetate 25:75% showed the non-polar nature of the active substance. This was substantiated by the report of 16 producer strains showing highest activity in ethyl acetate extract from trace to 26 mm, against *Bacillus subtilis* [35]. Similarly, biofilm forming marine bacterium D2 (*Pseudomonas tunicata*) isolated from the surface of the tunicate *Ciona intestinalis*, was found to produce a novel protein with bactericidal activity against wide variety of marine and medical bacterial isolates [36].

The production of compounds by the B3 strain could be categorized as exocellular as cell free culture media extracts showed activity against pathogens and so, there exists a possibility that the epibacterial strain B3 may produce antimicrobial substances of biomedical importance.

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REFERENCES

1. Jensen, P. and W. Fencial, 2000. Marine microorganisms and drug discovery current status and future potential. In Drugs from the sea(ed) N. Fusetani, Karger, Basel, Switzerland.
2. Burkholder, P.R., L.M. Burkholder and L.R. Almodovar, 1966. Antibiotic activity of some marine algae of Puerto Rico. Bot. Mar., 2: 149-156.
3. Trischman, J., D.M. Tapiolas, W. Fencial, R. Dwight and P.R. Jensen, 1994. Salimanide A and B anti-inflamatory depsipepdies from a marine Streptomycetes. J. Am. Chem. Soc., 116: 757-758.
4. Gerard, J., P. Haden, M.T. Kelly and A.J. Andersen, 1996. Loloatin B, a cyclic decapeptide antibiotic produced in culture by a tropical marine bacterium. J. Nat. Prod., 62: 80-85.
5. Imamura, N., M. Nishijima, Takadera, K. Adachi, M. Sakai and H. Sano, 1997. New anticancer antibiotics pelagiomicins producted by a new marine bacterium Pelagiobacter Variabillis. J. Antibiot., 50: 8-12.
6. Stierle, A., 2002. Montana invests-Building a better Montana, Montana Technology of the University of Montana, pp: 1-4.
7. Patterson, G.L. and C.M. Bolis, 1997. Fungal cell wall polysaccharides elicit an antifungal secondary metabolite (Phytoalexin) in the cyanobacterium Scytonema ocellatum. J. Phycol., 33: 54-60.
8. Wahl, M., P.R. Jensen and W. Fenical, 1994. Chemical control of bacterial epibiosis on ascidians. Mar. Ecol. Prog. Series., 110: 45-57.
9. Santhana Ramasamy, M. and A. Murugan, 2003 Chemical defense in ascidians *Eudistoma viride* and *Didemnum psammathodes* in Tuticorin, southeast coast of India: Bacterial epibiosis and fouling deterrent activity. Ind. J. Mar. Sci., 32(4): 337-339.
10. Strahl, E.D., W.E. Dobson, L.L. Lundie, 2002. Isolation and Screening of Brittlestar-Associated Bacteria for Antibacterial Activity. Curr. Microbiol., 44: 450-459.
11. Becerro, M.A., N.I. Lopez, X. Turon and M.J. Uniz, 1994. Antimicrobial activity and surface bacterial film in marine sponges. J. Exp. Mar. Biol. Ecol., 179: 195-205.
12. Murugan, A. and M.S. Ramasamy, 2003. Biofouling deterrent natural product from the ascidian Distaplia nathensis, Indian J. Mar. Sci., 32: 162-164.
13. Riguera, R., 1997. Isolating bioactive compound from marine organisms. J. Mar. Biotechnol., 5: 187-193.
14. Wright, A.E., 1998 Isolation of marine natural products. In: R.P.J. Cannell, editor. Methods in biotechnology, Vol. 4. Natural product isolation. New Jersey: Humana press Inc., pp: 305-408.
15. Slattery, M., J.B. McClintock and J. Heine, 1995. Chemical defenses in Antarctic soft corals: evidence for antifouling compounds. J. Exp. Mar. Biol. Eco., 190: 61-78.
16. Bernan, V.S., M. Greenstein and W.M. Maiese, 1997. Marine microorganisms as a source of new natural products. Adv. Appl. Microbial., 43: 57-89.
17. Thakur, N.L., A.N. Thakur and W.W. Muller, 2005. Marine natural products in drug discovery. Nat. Prod. Radiance, 4(6): 471-477.
18. Gil-Turnes, M.S., 1988. Antimicrobial metabolite produced by epibiotic bacteria their role in microbial competition and host defense. Ph.D. Dissertation, University of California - Sandiego, Lajolla, California.
19. Holmstrom, C., D. Rittschof and S. Kjelleberg, 1992. Inhibition of settlement by larvae of Balanus amphitrite and Ciona intestinalis by a surface-colonizing marine bacterium. Appl. Environ. Microbiol., 58: 2111-2115.
20. Spragg, A.M., M. Bregu, K.G. Boyd and J.G. Burgess, 1998. Cross-species induction and enhancement of antibiotic production by epiphytic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. Lett. Appl. Microbiol., 27: 142-146.
21. Al-Ogily, S.M. and E.W. Knight-Jones, 1977. Anti-fouling role of antibiotics produced by marine algae and bryozoans. Nature, 263: 728-729.
22. Mc Caffrey, E.J. and R. Endean, 1985 Antimicrobial activity of tropical and subtropical sponges. Mar. Biol., 89: 1-8.
23. Boyd, K.G., A.M. Spragg and J.G. Burgess, 1999. 0Screening of marine bacteria for the production of microbial repellents using a spectrophotometric chemotaxis assay. Mar. Biotechnol., 1: 359-363.

24. Lemos, M.L., A.E. Toranzo and J.L. Barja, 1985. Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. Microbiol. Ecol., 11: 149-163.
25. Shiba, T. and N. Taga, 1980. Heterotrophic bacteria attached to sea weeds. J. Exp. Mar. Biol. Ecol., 47: 251-258.
26. Patil, R., G. Jeyasekaran, S.A. Shanmugam and R. Jeyashalkila, 2001. Control of bacterial pathogens associated with fish disease by antagonistic marine Actinomycetes isolated from marine sediments. Indian J. Mar. Sci., 30(4): 264-267.
27. Chelossi, E., M. Milanese, A. Milano, R. Pronzanto and G. Riccardi, 2004. Characterization and antimicrobial activity of epibiotic bacteria from *Petrosia ficiformis*, (Porifera: Demospongiae). J. Exp. Mar. Biol. Ecol., 309: 21-33.
28. Nair, S. and U. Simidu, 1987. Distribution and significance of heterotrophic marine bacteria with antibacterial activity. Appl. Environ. Microbiol., 53(12): 2957-2962.
29. Burgess, J.G., E.M. Jordan, M. Bregu, A.M. Spragg and K.G. Boyd, 1999. Microbial antagonism, a neglected avenue of natural products research. J. Bacteriol., 70(1-3): 27-32.
30. James, S.G., C. Holmstrom and S. Kjelleberg, 1996. Purification and characterization of a novel antimicrobial protein from marine bacterium D2. Appl. Environ. Microbiol., 62(8): 2783-2788.
31. Maki, J.S., D. Rittschof, J.D. Costlow and R. Mitchell, 1988. Inhibition of attachment of larval barnacle, *Balanus amphitrite*, by bacterial surface films. Mar. Biol., 97: 199- 206.
32. Maki, J.S., L. Ding, J. Stokes, J.H. Kavouras and D. Rittschof, 2000. Substratum / bacterial interactions and larval attachment: Films and exopolysaccharides of *Halomonas marina* (ATCC 25374) and their effect on barnacle cyprid larvae, Balanus amphitrite Darwin. Biofouling, 16(2-4): 159-170.
33. Jeyasekaran, G., K. Jayanth and R. Jeya Shakila, 2002. Isolation of marine bacteria, antagonistic to human pathogens. Indian J. Mar. Sci., 31: 39-44.
34. Zheng, L., H. Chen, X. Han, W. Lin and X. Yan, 2005. Antimicrobial screening and active compound isolation from marine bacterium NJ6-3-1 associated with the sponge Hymeniacidon perleve. World J. Microbiol. and Biotechnol., 21(2): 201-206.
35. Anand, T.P., A.W. Bhat, Y.S. Shouche, U. Roy, J. Siddharth and S.P. Sarma, 2006. Antimicrobial activity of marine bacteria associated with sponges from the waters off the coast of South East India. Microbiological Res.,161(3): 252-262.
36. Zheng, L., X. Yan, J. Xu, H. Chen and W. Lin, 2005. *Hymeniacidon perleve*, associated bioactive *Pseudomonas* sp. NJ-6-3-1 Appl. Biochem. Microbiol., 41(1): 35-39.