JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. 7(4), p. 2879-2884

Isolation and Characterization of Antimicrobial Compound from *Bacillus* sp. Associated with Gastropods

S. Mary Josephine Punitha[1]*, N. Viju[1], S. Sharmin Vini[1], C.V. Sunjaiy Shankar[2], M. Michael Babu[1] and T. Citarasu[1]

[1]Center Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Kanyakumari, India.
[2]Department of Biotechnology, Udhaya College of Arts and Science, Velamodi, Kanyakumari, India.

(Received: 28 September 2013; accepted: 15 November 2013)

Screening of marine bacteria isolated from the marine environment has made a clear path for the identification of new antimicrobial metabolites. This study was carried out assess the antibacterial activity of gastropod associated bacteria. Gastropods were collected from west cost of India and a total of seven strains were isolated from the surface of it. The antimicrobial activity of the (EPS) Extracellular Polymeric Substances of the isolated strains were tested against four target bacteria *Galionella* sp., *Alteromonas* sp., *Pseudomonas* sp., *Klebsiella* sp. All the strains showed strong activity. The best active strain was selected and the active compound was characterized by HPLC analysis. The active strain was tentatively identified as *Bacillus* sp. based on morphological and biochemical characters. These bioactive compounds producing strains could be used as the source for the drug development in pharmaceuticals and industries.

Key words: Antimicrobial activity, Bioactive compounds; Surface-associated microorganism.

Bacteria are ubiquitous in the marine environment and colonize all marine habitats, from the deep oceans to the shallowest estuaries[1, 2]. Screening of marine bacteria isolated from the surface of marine algae and invertebrates has shown that a high percentage produce antimicrobial metabolites[3]. bacteria in biofilms formed on the surface of marine organisms have been documented to contain a high proportion of antibiotic producing bacteria than some other marine environment[4, 5]. Marine epiphytic bacteria, associated with nutrient rich algal surfaces and invertebrates, have also been shown to produce antibacterial secondary metabolites, which inhibit the settlement of potential competitors[6].

* To whom all correspondence should be addressed.
E-mail: punithapeter@gmail.com

A number of surface associated marine bacteria have also been found to produce antibiotics[7, 8]. Approximately 6,500 bioactive compounds were isolated from marine organisms[9]. Among the invertebrates, the molluscs are highly delicious seafood because of their nutritive value next to fin fishes and crustaceans. They are also very good source for biomedically important products[10]. Many classes of molluscs exhibits bioactive compounds like antitumour, antileukemic, antibacterial and antiviral properties have been reported world wide[11-14]. Among the molluscs, some animals exhibited pharmacological activities or other properties which are useful in the biomedical area.

The main objective of this study was to screen the antimicrobial activity of the bacteria associated with the gastropods collected from the Kanyakumari coastal waters. A study of this kind will improve our knowledge on the bioactivity of bacteria associated with marine invertebrates.

MATERIALS AND METHODS

Collection and Isolation of gastropod associated bacteria

Gastropods were collected from the Colachel coast (West Coast of India) and brought to the laboratory with a sterile container with seawater. In the laboratory, gastropods were gently washed with sterile seawater to remove loosely attached organisms and the surface associated bacteria was scrapped using a sterile nylon brush and suspended in 1ml filtered and sterilized sea water. This bacterial film suspension was serially diluted and spread on Zobell marine agar plates and incubated for 24 hours at 37°C for the development of colonies. After the period of incubation, the colonies were isolated based on colony morphology, and purified by repeatedly streaking on Zobell marine agar plates and maintained in slant for further works at 4 °C.

Isolation of extracellular polymeric substance and antimicrobial assay

Hundred microliter of culture broth was centrifuged at 5000 rpm for 15 minutes at 4°C. The cell pellets were discarded and the supernatant was mixed with equal amount of cold absolute ethanol. After one day incubation the precipitated EPS was collected and stored at 4°C.

Disc Diffusion (Kirby-Bauer) method was used for the antimicrobial activity. About 50 µl of the EPS were loaded on sterile paper discs (6 mm) and placed on the Zobell marine agar plates swabbed with target bacteria (*Galionella* sp., *Alteromonas* sp., *Pseudomonas* sp., *Klebsiella* sp). The diameter of inhibition zones were measured after incubation at 30°C for 24 h.

Thin-layer chromatography and antimicrobial activity of resolved compounds

The isolated EPS was characterized by thin layer chromatography. The samples were placed on silica gel plates and the solvent system used was benzene, acetic acid and methanol in the ratio of 1:1:3. Iodine crystals were used to visualize the spots and the Rf values were measured.

Spots appeared on the thin-layer chromatography were scraped and aseptically stored in sterile vials. From this, 1 g of the scraped compound along with silica gel was dissolved in 1 ml of distilled water. This was centrifuged at 5000 rpm for 15 minutes and the supernatant was used for antimicrobial assay. For this, 50 µl of supernatant was loaded on filter paper discs and were placed on the sterile Zobell marine agar plates swabbed with the test organism and incubated at 37°C for 24-48 hours to observe the zone of inhibition.

High performance liquid chromatography analysis

The active fraction of the compound present in the EPS of strain GP5 was used for HPLC analysis. Acetonitrite and water (50:50) was used as the mobile phase for the HPLC and the retention time was fixed at 1 ml / min.

RESULTS AND DISCUSSION

In this study, seven (GP1, GP2, GP3, GP4, GP5, GP6 and GP7) strains were isolated from the surface of gastropods and identified based on morphological and biochemical characteristics, all the seven strains were Gram-negative and non-motile, additional biochemical test results are given in Table 1. Isolated strains were tentatively identified according to Bergey‘s manual of determinative bacteriology.

The (EPS) extracellular polymeric substances isolated from all the seven bacterial strain were tested for the antimicrobial activity against four strains (*Galionella* sp., *Alteromonas* sp., *Pseudomonas* sp., and *Klebsiella* sp). Strains GP1 showed inhibitory activity against all the four target bacteria. Strain GP3, GP4, GP5 and GP7 inhibited the growth of three (*Galionella* sp., *Alteromonas* sp., and *Klebsiella* sp) bacterial strains. It did not show inhibitory activity against *Pseudomonas* sp. Strain GP2 and GP6 inhibited the growth of *Pseudomonas* sp and *Alteromonas* sp., did not inhibit the growth of *Galionella* and *Klebsiella* sp. and strain GP6 showed maximum inhibitory zone of 12 mm against *Alteromonas* sp. (Table 2).

For the characterization of active compounds, the EPS was loaded on silica gel plates. Thin layer Chromatogram showed the presence of a single spot in all the strains. The Rf values were measured and are given in the Table 3. The possible bioactive compounds were eluted and tested for their antimicrobial activity against the target bacteria. Strain G5 showed good activity against *Klebsiella* sp (10 mm). and *Alteromonas*

sp.(12 mm). It did not show any activity against *Pseudomonas* sp. and *Galionella* sp (Table 4).

The EPS of active strain GP5 was partially characterized through HPLC analysis. The HPLC spectrum showed six peaks and the peaks were observed at the retention time of 1.28, 1.55, 1.65,

Table 1. Biochemical and physiological characteristics of the bacterial strains isolates used in present study

S. No	Biochemical and physiological Test	Organisms						
		GP1	GP2	GP3	GP4	GP5	GP6	GP7
1	Gram staining	+	-	-	-	+	-	-
2	Morphology	rod	cocci	cocci	cocci	rod	cocci	Cocci
3	Motility	+	-	-	-	-	+	+
4	Indole production	-	-	-	-	+	-	+
5	Methyl Red	+	+	-	+	-	+	+
6	Voges – Proskauer	-	-	-	+	-	+	+
7	Citrate utilization	+	+	+	+	+	+	+
8	Starch Hydrolysis	-	+	+	+	+	+	-
9	Urea Hydrolysis	-	-	+	-	+	+	-
10	Utilization of CHO							
	Glucose	+	+	+	+	+	+	+
	Fructose	-	+	+	-	-	+	+
	Lactose	-	-	-	+	-	-	-
	Manitol	-	-	-	+	+	+	+
	Sorbitol	-	-	-	-	-	-	-
11	Catalase	-	+	+	+	+	+	+
12	Oxidase	+	+	-	+	-	+	+
13	Nitrate Reduction	+	+	-	+	+	-	+
14	Casein Hydrolysis	-	+	+	+	-	+	+
15	Gelatin Hydrolysis	+	+	+	+	-	-	-

Table 2. Antibacterial activity of EPS against target Bacteria

S. No	Isolated Strains	Zone of inhibition (mm)			
		Pseudomonas sp.	*Klebsiella* sp.	*Alteromonas* sp.	*Galionella* sp.
1	GP1	9	11	10	10
2	GP2	9		10	
3	GP3		9	11	10
4	GP4		9	10	9
5	GP5		9	11	9
6	GP6	9		12	
7	GP7		9	9	10

Table 3. RF value of compounds observed in the TLC

S. No	Isolated Strains	RF Values (cm)
1	GP1	0.82
2	GP2	0.76
3	GP3	0.79
4	GP4	0.82
5	GP5	0.85
6	GP6	0.73
7	GP7	0.75

Table 4. Antibacterial activity of compounds recovered from the TLC

S. No	Target Bacteria	Zone of inhibition of strain GP5 (mm)
1	*Pseudomonas* sp.	-
2	*Klebsiella* sp.	10
3	*Alteromonas* sp.	11
4	*Galionella* sp.	-

2.12, 2.26 and 5.06 min respectively. The maximum peak was observed at the retention time of 2.12 min and the height of the peak is 2674 mAU (Fig. 4).

The bacterial associations with marine plants and animals, which range from casual encounters to obligate symbioses, provide unique opportunities for bacterial adaptation. It is proposed that some of these adaptations would not be selected for the absence of environmental parameters associated with the host, and that these adaptations can include the biosynthesis of unique metabolic products[15]. A less understood chemically mediated interaction is the bacterial production of bioactive compounds which may enhance their ability to compete with other organisms for space and nutrients. Numerous studies have demonstrated that bacteria produce active compounds against other microorganisms as well as against higher organisms. The variation in the activity of the bacterial strains against different target organisms suggested that different antibacterial compounds are produced by each species and thereby it led to the conclusion that the diversity and numbers of bacteria on marine living surfaces may also accommodate surface associated microorganisms that produce antimicrobial agents. The high proportion of antimicrobial producing strains may be associated with an ecological role, playing a defensive action to maintain their niche, or enabling the invasion of a strain into an established microbial community.

Result of the present study shows the antibacterial effect of each isolate upon the growth of four different pathogenic strains and the inhibitory activity against almost all the pathogens ranging from 9 mm to 12 mm. In this context the surface associated bacteria showed some specificity in their antimicrobial activity against target strains, therefore they may contain some metabolites potentially able to inhibit the attachment of other microorganism.

The EPS of strain G5 was characterized by TLC and HPLC analysis. The results of TLC demonstrated that the EPS produced by bacterial isolates could have single antimicrobial metabolites. The HPLC analysis showed six peaks and the maximum peak was observed at the retention time of 2.12 minutes. This indicates that the compounds recovered from the TLC were not

Fig. 1. Antibacterial activity of EPS isolated from strain GP5 against *Alteromonas* sp (b) Antibacterial activity of EPS of strain GP1 against *Alteromonas* sp. 50 µl of EPS was loaded on antimicrobial assay discs (6mm). Inhibitory activity was observed after 24 hours of inhibition at room temperature

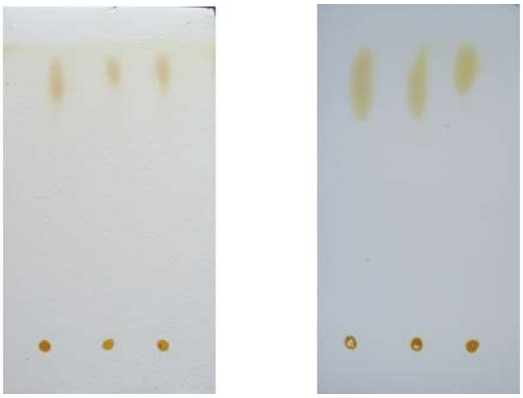
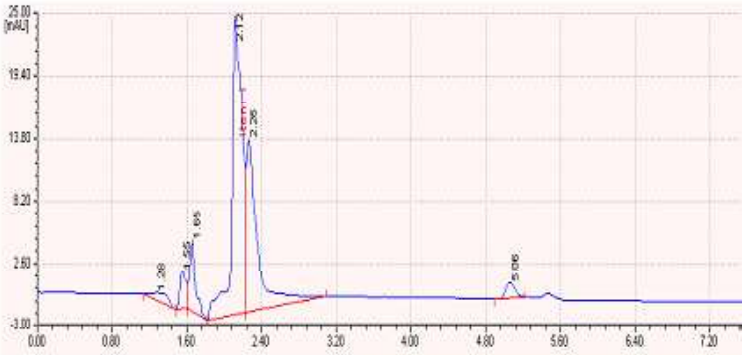


Fig. 2(a). Thin layer chromatography analysis of the EPS of strain GP5. (b) Thin layer chromatography analysis of the EPS of strain GP1. The EPS was loaded on silica gel plate and the solvent system used was benzene, acetic acid and methanol in the ratio of 1:1:3.

Fig. 3(a). Antibacterial activity of TLC recoverd compound of strain GP5 against *Alterompnas* sp.(b) Antibacterial activity of TLC recoverd compound of strain GP 1 against *Klebsiella*sp. 50 µl of concentrated TLC recovered compound was loaded on antimicrobial assay discs (6mm). Inhibitory activity was observed after 24 hours of inhibition at room temperature

completely purified.

In this study, strain GP5 was tentatively identified as *Bacillus* sp. A variety of antimicrobial compounds are produced by members of the genus *Bacillus*, many of these identified as peptides, lipopeptides and phenolic derivatives[16]. A wide range of antimicrobial substances produced by *Bacillus* spp. isolated from arthropods were recently described, including aromatic acids, acetylamino acids (amino acid analogs), and peptides[17]. In conclusion, results of the present study shows that bacteria associated with gastropods have strong antibacterial activity. Since the active compounds present in the isolated strains prevent the growth of target bacteria it could be used as the source for the development of drugs from marine resources.



Parameter | Result | Group Result | Sample Inf

ID	Name	RT(min)	Height	Area	Conc	Half Width(s)	Res	Theo Plate	Tail Fact
1		1.278	69	1033.3	2.6246	14.97	0.00	145.25	0.97
2		1.553	336	1503.4	3.8188	4.47	1.00	2402.11	0.89
3		1.655	633	2640.8	6.7079	4.17	0.83	3136.29	1.53
4		2.117	2674	20521.7	52.1264	7.67	2.75	1516.10	0.70
1	Item 1	2.263	1552	12790.4	32.4884	8.24	0.65	1503.21	11.34
6		5.063	137	879.4	2.2339	6.42	13.48	12399.67	1.31
		xÜ¼Æ:	iÆ=5401	iÆ=39369.0	iÆ=100.0000				

Fig. 4. HPLC analysis of the EPS of best active strain GP5. Acetonitrile and water (1:1) was used as solvent system

ACKNOWLEDGMENTS

The authors thank University Grant Commission for financial assistance through UGC major project scheme (Ref. F. No: 39-294/2010 SR).

REFERENCES

1. Anand, Y.H. and Friedman, R.J. Concise review of mechanisms of bacterial adhesionto biomaterial surfaces. *J. Biomed. Mater. Res. Part B.*, 1998; **43**(3): 338-348.
2. Rheinheimer, G., Aquatic microbiology; Wiley: Chichester; New York, NY, USA, 1992.
3. Burgess, J.G., Jordan, E.M, Bregu, M., Means–Spragg. and Boyd, K.G. Microbial antagonism of natural products research. *J.Biotechnology*., 1999; **70**: 27-32.
4. Lemos, M.L., Toranzo, A.E. and Barja, J.L. Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. *Microb. Ecol*., 1985; **11**: 149-163.
5. Anand, T.P., Bhat, A.W., Shouche, Y.S., Roy, U., Siddharth, J. and Sarma, S.P. Antimicrobial activity of marine bacteria associated with sponges from the waters off the coast of South East India. *Microbiol.Res*., 2006; **161**: 252-62.
6. Bernan, V.S., Greenstein, M. and Maise, W.M. Marine microorganisms as a source of new natural products. *Adv. Appl. Microbiol*., 1997; **43**: 57-90.
7. Holmstrom, C. and Kjelleberg, S. Marine *Pseudoalteromonas*species are associated with higher organisms and produce biologically active

extracellular agents. *FEMS Microbiol. Ecol*., 1999; 30: 285-293.

8. Hans-Peter, G., Andrea, S., Michael, B., Meinhard, S. and Thorsten.B. Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. *FEMS Microbiol Ecology* ., 2004; **1619**: 1-11.
9. Somayajulu, B.L.K. Bioactive Agents from the Ocean Biota. In: Ocean Science Trends and Future Directions (Kamboj VP, ed.). *Indian National Science Academy, New Delhi,* India, 1999; **6**: 197-227.
10. Shenoy, A.S. Octopus a delicay in Japan. *Sea Food Exp*., 1988; **20**: 21-25.
11. Kamiya, H., Muromota, K., Sakai, G.K., Endo, Y.M. and Yamamzaki, M.J. Purification and characterization of an antibacterial and anticoplastic protein secretion of a sea hare. *Aplysiajuliana*. *Toxicon*., 1989; **27**:1269-1277.
12. Pettit, G.R., Kamano, Y., Hurald, C.L., Tuinman, A.A. and Boethner, F.E.X. The isolation and structure of a remarkable marine animal anti neoplastic constituent: Dolostatin 10. *J. Am. Chem. Soc*., 1987; **109**: 6883-6885.
13. Anand, T.P., Rajaganapathi, J. and Edward, J.K.P. Antibacterial activity of marine molluscs from Portonovo region. *Indian J. Mar. Sci*., 1997; **26**: 206-208.
14. Rajaganapathi, J., Thyagarajan, S.P. and Edward, J.K.P., Study on Cephalopodink for antiretroviral activity. *J. Exp. Biol*., 2000; **38**: 519-520.
15. Jensen, P.R. and W. Fenical.: Marine microorganisms and drug discovery: current status and future potential. In: Drugs from the sea, (Fusetani, N, ed). Basel: Karger, 1998: pp 6-29.
16. Nakano, M.M. and Zuber, P. Molecular biology of antibiotic production in Bacillus. *Crit. Rev. Biotechnol*., 1990; **10**: 223–240.
17. Gebhardt, K., Schimana, J., Muller, J., Fielder, H. P., Kallenborn, H.G., holzenkampfer, M., Krastel, P., Zeeck, A., Vater, J., HOltzel, A., Schmid, D.G., Rheinheimer, J. and Dettner, K. Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. *FEMS. Microbiol. Lett*., 2002 ; **217**: 199–205.