academicJournals

Vol. 8(41), pp. 3617-3624, 8 October, 2014
DOI: 10.5897/AJMR2014.7022
Article Number: 8A209F348132
ISSN 1996-0808

http://www.academicjournals.org/AJMR

AfricanJournalofMicrobiology Research

Full Length Research Paper

Chemical characterization and bioactivity evaluation of bacteriocin from marine biofilm-forming bacteria

Sengol Jenifer S.[1], V. Balasubramanian[2] and R. Rajaram[2]*

[1]Department of Biotechnology, Seethalakshmi Ramaswami College, Tiruchirappalli-620 002, Tamilnadu, India.
[2]Department of Marine Science, Bharathidasan University, Tiruchirappalli-620 024, Tamilnadu, India.

Received 18 July, 2014; Accepted 22 September, 2014

The marine biofilm bacteria were isolated on polyvinyl chloride (PVC) sheet immersed from offshore platform of Central Electrochemical Research Center (CECRI), Tuticorin, Tami Nadu. The isolated marine biofilm bacteria such as *Pseudomonas* sp., *Aeromonas* sp., *Enterobacter* sp., Bacillus spp., *Flavobacterium* sp., *Micrococcus* sp. and *Cytophaga* sp were identified up to generic level. The *Pseudomonas* sp. was the dominant, primary biofilm forming bacteria. The cell free crude extract of single (*Aeromonas* sp.) and co-cultured (*Aeromonas* sp. + *Enterobacter* sp.) system showed the significant repellent activity against *Pseudomonas* sp. The crude fractions were characterized by FTIR for functional group identification and GC-MS for compound identification. The results of FTIR shows different peaks like 3302.16, 2816.56, 1227.44 and 2724.02 cm^{-1} indicating the presence of bromine, amine, and phenolic, hydroxyl, amino, carbonyl and phosphoryl functional groups. It was also noted that mixed culture system produced increased level of bacteriocin as compared to single culture system. The chemical characterizations of the inhibitory molecules such as nonane, is common to both single as well as co-culture system. The higher inhibitory activity observed in co-culture system is due to the presence of nonadecane, heneicosane and cholestane compounds. The natural biogenic compounds from the marine bacteria studied have the potential to be used as a substitute to commercial biocides for anti-microfouling.

Key words: Anti-biofilm activity, bacteriocin, nonane, bioactivity, bioremediation.

INTRODUCTION

Marine structures such as ships, marine platforms, offshore rigs and jetties are under constant attack from the marine environment by fouling organisms. These structures need to be protected from the influences of the key elements of the marine environment such as saltwater, temperature fluctuations and biofouling (Chambers et al., 2006). Biofouling is one of the major significant problems and ubiquitous in the marine environment. It is a natural process of colonization of submerged surfaces, either living or artificial, involving a wide range of micro and macro organisms

*Corresponding author. E-mail: drrajaram69@rediffmail.com. Tel: 0091-9842874661. Fax: 0091-431-240745.

Figure 1. The Biofilm development rack.

(Briand, 2009). The attachment of microorganisms like bacteria, algae, diatoms and fungi are called microfouling (biofilm), which initiates the macrofouling. The problem of biofouling is so severe that worldwide the expenditure incurred on antifouling measures alone is approximately US$ 6.5 billion a year (Bhadury and Wright, 2004). Application of antifouling coatings like self- polishing copolymer antifouling paints (SPCs) with tributyl tin (TBT) as biocide and copper-based paints were widely employed for controlling fouling on marine structures (Chamberset al., 2006; Claisse and Alzieu, 1993; Batley et al., 1994). However, the toxicity of TBT and copper to marine organisms cause bioaccumulation of metal, imposex, etc. Nowadays, natural products from marine organisms can be used as replacements for the chemicals commonly used in antifouling coatings (Clare, 1996). Bacteriocins are antimicrobial compounds produced by bacteria that can kill closely related strains of the same species. Marine bacteria, primarily belonging to the *Pseudoalteromonas* genus, the *Vibrionaceae* family, and the *Roseobacter clade*, excrete compounds that can reduce bacterial biofilm formation and settlement by microorganisms on surfaces suggesting their suitability as antifouling bacteria (Bowman, 2007; Dobretsov and Qian, 2004). The chemotaxis activity of the bacteriocins toward the microfouling bacteria to prevent the initial biofilm formation, accordingly the prevention of biofilm would be a safe and effective way to prevent the macrofouling through eco-friendly (Armstrong et al., 2000). The main purpose of the present study was to screen the antifouling potential of marine bacterial metabolites against primary biofilm forming bacteria. Finally, chemical characterization of bacteriocin obtained from the co-culture of *Aeromonas* sp. and *Enterobacter* sp. was done.

MATERIALS AND METHODS

Marine biofilm bacterial strain

Polyvinyl chloride (PVC) sheet was cut into the dimension of 12″ ×12″ and the sheets were degreased using acetone and mounted on a wooden rack having the total size of 75″ × 15″, using brass bolt and nut. The rack was immersed at 2 m depth from the mean surface seawater below the offshore platform of Central Electro Chemical Research Institute (CECRI) at Tuticorin unit, Tamilnadu, India for 24 h (Figure 1). After 24 h, the biofilm was scrapped from PVC sheets using sterile brush and transferred to glass tube containing sterile seawater. Further the scrubbed biofilm samples was serially diluted and spread on Zobell Marine Agar and the plates were incubate up to 24 h at 37°C. The isolated biofilm bacteria were characterized using various morphological and biochemical tests (Allegrucci and Sauer, 2007; Balasubramanian et al., 2012).

Bacteriocin activity

For bacteriocin production, the isolates were inoculated by means of (i) single cell culture by *Aeromonas* sp. and co-culture by *Aeromonas*sp + *Enterobacter* sp. were prepared in Zobellmarine broth and incubated at 37°C for 24-48 h in orbital shaker at 120 rpm over night. The growth kinetics of the single as wells co-culture systems has been monitored by taking the OD_{600} starting from 0, 1, 2, 3, 4, 5, 10, 15, 20 and 24 h respectively (Sher et al, 2011). The bacteriocins were extracted after 48 h of culture, because the production rate is high during the exponential phase and late stationary (Hammami et al., 2009).

The cultures were centrifuged at 6000*g* and the supernatant was precipitated with ammonium sulfate at 80% saturation under chilled conditions for 18-24 h. The precipitated bacteriocins were collected by centrifugation (10,000 *g*, 30 min) and the pellet was dissolved in 50 mMTris-HCl (Lili et al., 2006). The agar well diffusion method was used for detection of bacteriocin activity in crude cell-free supernatants from single and co-culture systems against primary biofilm forming bacteria *Pseudomonas* sp. All the plates were incubated at 37°C for 24 h; the zone of inhibition was observed (Todorov and Dicks, 2005).

Analysis of natural products

The qualitative analysis by FTIR (Model RX) spectrometry has enabled us to make an identification of functional groups present in the sample of the crude extracts. The crude extracts were subjected to column chromatography over silica gel (Merck, mesh size 100-200µm) and eluted with an *n*-hexane/ethyl acetate combination (8:2). The major bioactive fractions of *n*-hexane-ethyl acetate were repeatedly run over the silica gel and these crude fractions were separated and subjected to GC-MS analysis. Analysis of natural

Table 1. The biochemical characteristics of microbial isolates.

Biochemical parameters							Suggested Genera
Gram Staining	Motility	Indole	Oxidase	CH_2O	Penicillin sensitivity	Pigmentation	
G^-	+	-	-	-	-	-	***Pseudomonas* sp.
G^-	+	+	+	AG	-	-	**Aeromonas* sp.
G^-	+	+	-	AG	-	-	**Enterobacter* sp.
G^+	+	+	-	-	-	-	**Bacillus* sp.
G^+	-	-	-	-	-	-	**Micrococcus* sp.
G^-	+	-	-	-	-	Orange	**Flavobacterium* sp.
G^-	+	-	-	-	+	Yellow	**Cytophaga* sp.

*** Dominated groups; ** Moderate; * presence; *AG* – acid and gas.

Table 2. The seawater physicochemical parameters for the study area.

Parameter	Tuticorin - Open sea
Salinity (‰)	35.00
pH	7.8
Dissolved oxygen (mg/l)	4.05
Inorganic phosphate (µmol/l)	0.725
Nitrite (µmol/)	0.0150
Nitrate (µmol/)	3.37
Ammonia (µmol/)	2.25
Calcium (mg/l)	400
Magnesium (mg/l)	1275
Total phosphorus (µmol/l)	3.27

products was carried out with a GC-MS (SHIMADZU, QP 2010) for identification of the mass proportion of active fractions (Bhattarai et al., 2007).

Crystal violet binding assay

The overnight culture of biofilm bacteria *Pseudomonas* sp. was diluted to about 1×10^6CFU/ml with fresh sterile medium. The antifouling potential of each metabolite from single and co-culture system was tested against *Pseudomonas* sp. using 96-well microtiter, the plates were gently shaken to permit growth and encourage biofilm formation. After an incubation period of 24 h, the plates were washed with PBS buffer, air dried and stained with 0.2% crystal violet solution. The plates were then again washed thoroughly to remove any unbound crystal violet dye. Ethyl alcoholwas (99.0%) then added to the wells in order to elute the crystal violet dye adhering to the biofilm (Thenmozhi et al., 2009).

RESULTS AND DISCUSSION

Seawater characteristics and bacterial identification

The isolated and identified marine bacterial isolates are *Pseudomonas* sp., *Aeromonas* sp., *Enterobacter* sp., *Cytophaga* sp., *Micrococcus* sp., *Bacillus* sp., *Flavobacterium* sp. Among the isolates, *Pseudomonas* sp. is found in dominant and initial biofilm forming bacteria followed by *Aeromonas* sp. and *Enterobacter* sp. (Table 1). The physico-chemical characters of the Tuticorin coastal water samples were found in normal (Table 2).

Growth kinetics of the bacteriocin producing biofilm bacteria

The growth pattern among the single and co-culture system, the mixed cultures of *Aeromonas* sp. and *Enterobacter* sp. showed increased growth rate. In both culture, bacteriocin production starts high during the exponential phase (12 h) and increased bacterial growth rate was observed in stationary phase (Boe, 1996; Bizani and Brandelli, 2002). The high bacteriocin production in exponential phase is due to increased bacterial growth rate as observed in stationary phase (Boe, 1996). It was also noted that co-cultures system shows significant increased growth rate where as single culture system shows the decrease level of growth rate. The bacteriocin from mixed culture system shows significant antagonistic activity against marine biofilm forming *Pseudomonas* sp. (Figure 2) is due to presence of more antibiofilm compounds in comparison with single cell culture system (Table 3). The prevention of the bacterial adhesion at the very initial stage, can considerably reduce the risk of further biofilm formation.

Bacteriocin activity

Bacteria from marine environment are known for their rich source of bioactive molecules, but the reports are scanty for their antibiofilm metabolites (Thenmozh et al., 2011; Bakkiyaraj and Pandian, 2010; Nithya et al., 2010). Moreover, several studies suggest many marine bacteria are capable of producing novel antibiofilm compound(s)

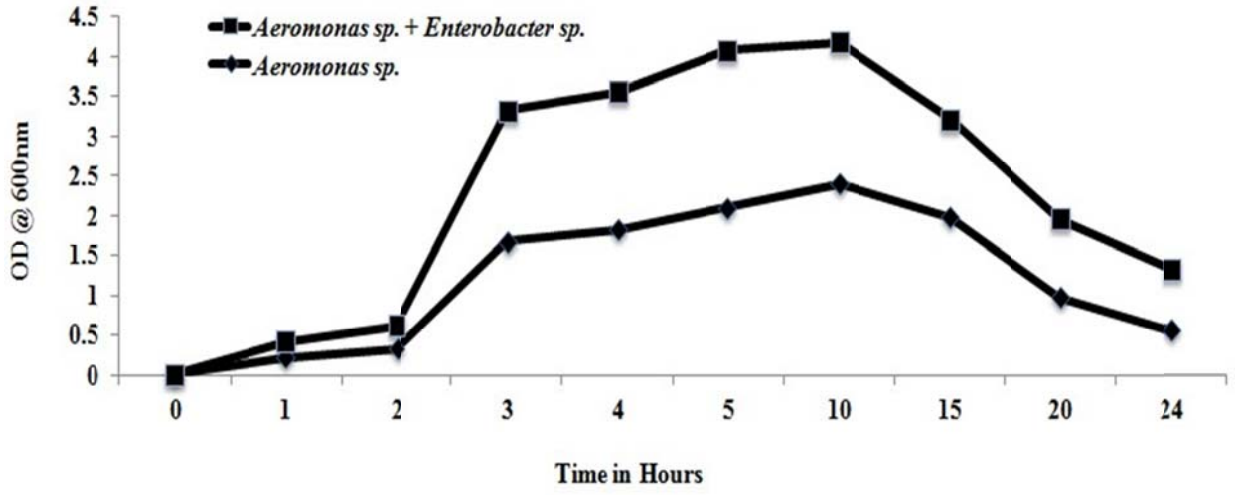

Figure 2. The growth kinetics of bacteriocin producing marine bacteria.

Table 3. FTIR spectrum of the single and co culture extracts.

S/N	Frequency (cm^{-1})	Functional group assignment	References
Single culture			
1	3302.16	H, Free NH, CH stretching vibrations,=C–H	Dumas and Miller[34]
2	2816.56	C–H of CH_3,CH_2,CH	
3	2084.11	C–C=C–C=CH stretching vibrations of $–CH_3>CH_2$	Guo[36]
4	1354.57	C □□O	Stuart[35]
Co culture			
1	3428.16	O–H AND N–H stretching vibration	Guo[36]
2	2094.80	C–C=C–C=CH	
3	2811.70	C–H stretching vibrations of $–CH_3>CH_2$	Stuart[35]
4	1601.07	C–N stretching	Guo[36]
5	1353.34	C-H of CH3, CH2 groups and ɣs C-O of COO− groups	Wolkers[37]
6	1227.44	C–N stretching	Yee *et al*[38]

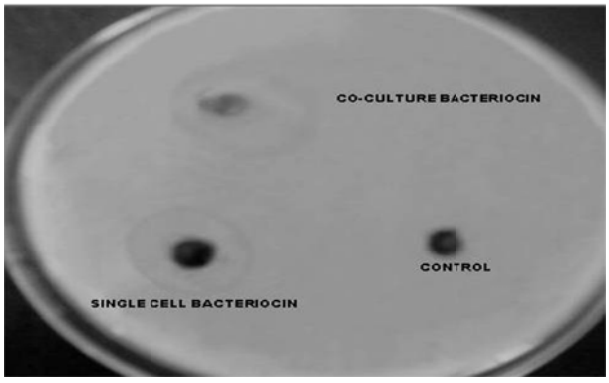

Figure 3. The bacteriocin activity against *Pseudomonas* sp.

which have not been tapped from terrestrial environment (El-Gendy et al., 2008; Selvin et al. 2009). In the present study, bacteriocin extracts obtained from marine biofilm bacteria were used to test against primary biofilm forming bacteria *Pseudomonas* sp. by using agar well method. The zone of inhibition was high in bacteriocin obtained from co-culture system as compared to single culture (Figure 3). A few anti-biofilm metabolites secreted by bacteria and devoid of antibacterial activity against free living cells were previously reported as in *E. coli* strains producing group II capsules that release a soluble polysaccharide in their environment, preventing biofilm formation by a wide range of Gram-positive and negative bacteria (Valleet al., 2006).

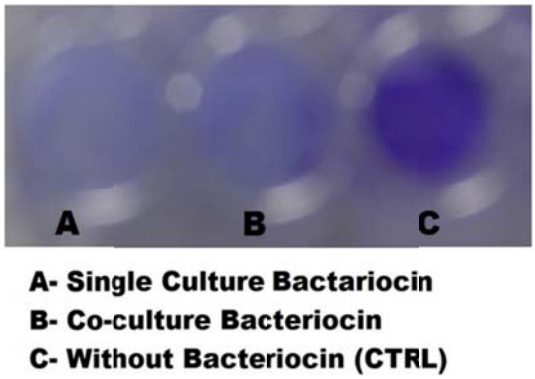

Figure 4. Biofilm stained with crystal violet.

Crystal violet binding assay

The 96-well polystyrene microtiter plate assays is a simple means of testing bacterial biofilm formation; it is a rapid and quantitative method which permits the direct quantification of adhered marine bacteria in a microtiter plate. This basic assay can be adapted readily to study several aspects of biofilm formation (Jianget al., 2011). The result of the 96-well reveals the bacteriocins from both culture systems shows significant decreased biofilm formation (Figure 4), the dark well indicates biofilm positive and light well shows biofilm negative. The well contains the culture of *Pseudomonas* sp. along with bacteriocin showing light colour when dye with crystal violet is due to the presence of anti-adherent compound in bacteriocin. The culture of *Pseudomonas* sp. alone in the well shows dark colour when dye with crystal violet indicate the biofilm formation in 96-well (Thenmozhi et al., 2011).

FTIR spectrum of the bacterial metabolites

FT-IR spectroscopy defines the chemical composition of single as well as co-culture system of the marine bacterial isolates exhibited various active metabolites (Figure 5). The functional groups spectral region (4000-400cm^{-1}) obtained from single culture systems exhibits four major peaks whereas six major peaks are observed in co-culture systems (Table3).The higher antibiofilm activity against *Pseudomonas* sp. observed in the bacteriocin of co-culture system is due to the presence of additional functional peaks like 1227.44 and 2724.02 cm^{-1} could have been active against the marine biofilm bacteria.

GC-MS analysis of the crude cell extract

The results pertaining to GC-MS analysis leads to theidentification of number of compounds from hexane and ethyl acetate extract (8:2) of single culture (*Aeromonas* sp.) and co-culture strains (*Aeromonas* sp. + *Enterobacter* sp.) clearly revealed the presence of several organic metabolites acts as a anti-biofilm in nature. Chemical characteristics of active fraction on the basis of spectral data by GC-MS were found to be a mixture of various biogenic compounds. The characterization of the inhibitory molecules exhibited in secondary metabolites such as nonane, pentadecane and hexadecane is common to both single as well as co-culture system. The compounds 3-eicosanone, cyclohexane, undecane, octadecane and docosane were exhibited in single culture system likewise iron, nonadecane, heneicosane, cholestane were exhibited in co-culture system (Table 4 and Figure 6). The higher antibiofim activity associated with co-culture's bacteriocin is due to the presence of iron, nonadecane, heneicosane and cholestane.

Sponge associated marine bacteria produced octadecaneand their antibacterial and antilarval- settlement activity was for possible new sources of less toxic bioactive antifoulants (Dash et al., 2009). The chloroform extract of *Andrographis paniculata* exhibits the octadecane, hexacosane, eicosane, heptadecane observed to be active against the opportunistic and pathogenic Gram negative bacteria (Roy et al., 2010). Previous report showed antimicrobial activity of nonane extracted from individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils (Pascalet al., 2002).

In the present study, the bacteriocin produced in the co-culture system shows good anti-biofilm activity against Gram negative pioneering marine biofilm forming bacteria *Pseudomonas* sp. Accordingly, the Gram negative bacterium *Pseudomonas* sp. was found to be the pioneer bacteria to colonize the marine structure. Marine bacterium *Pseudomonas rhizosphaerae* could produce potent antibacterial and antilarval secondary metabolites. The antibacterial compounds including cyclo-(Tyr-Pro), cyclo-(Tyr-Ile), cyclo-(Phe-Pro), cyclo-(Val-Pro), 3-phenyl-2-propenoic acid and uracil had various antibacterial activities towards five marine fouling bacteria (Qi et al., 2009). Our prime purpose was not only the identification of antibacterial activity, but also to screen the anti-biofilm activity expressed by marine bacteria producing bacteriocins. The present study suggests that marine bacterial isolates are the potential source for isolation of anti-biofilm agents as they have broad spectrum of activity against the target organisms. These metabolites can be further exploited for the use of antifouling compounds against biofouling through eco-friendly manner.

Conflict of interest

The author(s) have not declared any conflict of interests.

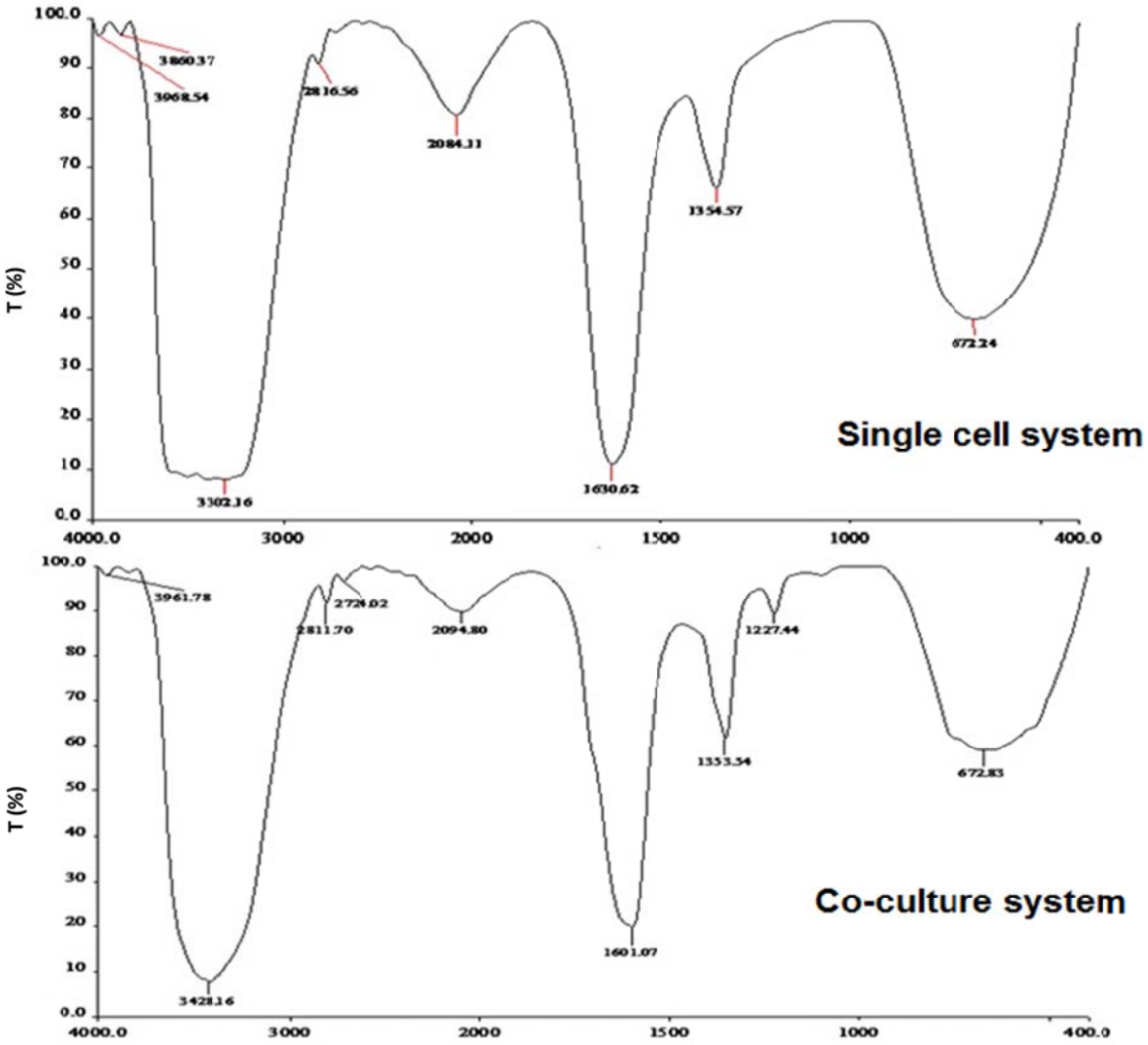

Figure 5. The FTIR spectrum of crude metabolites.

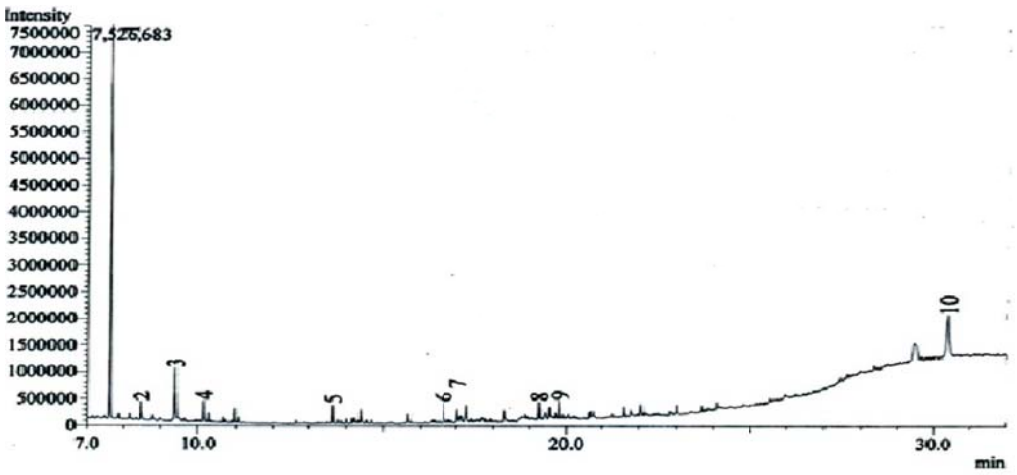

Figure 6a. The GC–MS spectrum of antibiofilm compounds in single culture systems.

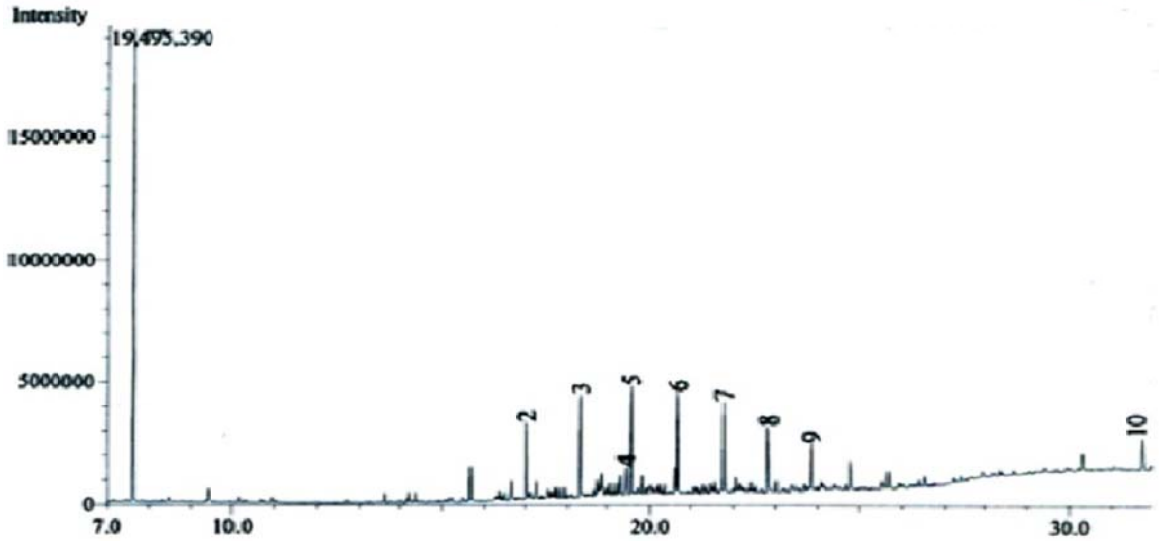

Figure 6b. The GC–MS spectrum of antibiofilm compounds in co-culture systems.

Table 4. Chemical compounds characterized by GC–MS (QP 2010 SHIMADZU).

Peak	R. Time	Area	Height	Compound	Base (m/z)
Single culture					
1	7.607	9849940	7375197	Nonane	57.05
2	8.478	599376	325846	3-Eicosanone	85.05
3	10.157	552440	368021	Undecane	57.05
4	13.651	545566	333792	Octadecane	57.00
5	16.641	515295	323444	Docosane	57.05
7	17.033	406779	216232	Pentadecane	57.10
7	19.261	490355	304833	Hexadecane	57.10
8	19.808	551578	328270	Docosane	57.00
9	30.392	3012664	684368	4-Bromo-2,6-bis(3,5-di-tert-butly-4-hydroxy)	57.05
Co-culture					
1	7.607	25707907	19331280	Nonane	57.05
2	17.027	4597973	3069066	Pentadecane	57.05
3	18.314	6196065	4086885	Hexadecane	57.05
4	19.407	2213676	1078919	Pentadecane	57.05
5	19.532	6331828	4343250	Iron	57.05
6	20.686	6321679	4070423	Nonadecane	57.05
7	21.783	5025673	3586753	Nonadecane	57.05
8	22.827	4050007	25662719	Nonadecane	57.05
9	23.824	2445399	1773641	Heneicosane	57.05
10	31.769	2561988	1202010	Cholestane	81.05

ACKNOWLEDGEMENTS

The authors are also thankful to Dr. G. Subramanian and S. Palanichamy, CECRI (OPMEC), Tuticorin Unit – 628 004, Tamil Nadu for providing facilities to conduct the biofilm formation studies in OPMEC, CECRI, Tuticorin and Dr. R. BabuRajendran, Department of Environmental Biotechnology, Bharathidasan University for GC-MS

analysis of bacteriocin samples.

REFERENCES

Allegrucci M, Sauer K (2007). Characterization of colony morphology variants isolated from Streptococcus pneumoniae biofilms. J. Bacteriol. 189:2030-2038.

Armstrong E, Boyd KG, Pisacane A, Peppiatt CJ, Burgess JG (2000). Marine microbial natural products in antifouling coatings. Biofouling 16:215-224.

Bakkiyaraj D, Pandian SK (2010). *In vitro* and *in vivo* antibiofilm activity of a coral associated actinomycete against drug resistant *Staphylococcus aureus* biofilms. Biofouling 26:711-717.

Balasubramanian V, Palanichamy S, Rajaram R (2012). Development of polyvinyl chloride biofilms for succession of selected marine bacterial populations. J. Environ. Biol. 33:57-60.

Batley GE, Chapman JC, Wilson SP (1994). Environmental impacts of antifouling technology. In: Kjelleberg S, Steinberg P (eds) Biofouling: Problems and Solutions. UNSW, Sydney. pp. 49-53.

Bhadury P, Wright PC (2004). Exploitation of marine algae: biogenic compounds for potential antifouling applications. Planta 219:561-578.

Bhattarai HD, Ganti VS, Paudel B, Lee YK, Lee HK, Hong Y-K, Shin HW (2007). Isolation of antifouling compounds from the marine bacterium *Shewanella oneidensis*. World J. Microbiol. Biotechnol. 23:243-249.

Bizani D, Brandelli A (2002). Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8 A. J. Appl. Microbiol. 93:512-519

Boe J (1996). Evaluation of optimum production for bacteriocin from *Lactobacillus* sp. JB 42 isolation from Kimichi. J. Microbiol. Biotechnol. 6:63-67.

Bowman JP (2007). Bioactive compound synthetic capacity and ecological significance of marine bacterial genus *Pseudoalteromonas.* Mar. Drugs 5:220-241.

Briand JF (2009). Marine antifouling laboratory bioassays: an overview of their diversity. Biofouling 25:297-311

Chambers LD, Stokes KR, Walsh FC, Wood RJK (2006). Modern approaches to marine antifouling coatings. Surf. Coat. Technol. 201:3642-3652.

Claisse D, Alzieu C (1993). Copper contamination as a result of antifouling paint regulations?. Mar. Pollut. Bull. 26: 395-397.

Clare AS (1996). Marine natural product antifoulants: status and potential. Biofouling 9:211-229.

Dash S, Jin C, Lee OO, Xu Y, Qian PY (2009). Antibacterial and antilarval-settlement potential and metabolite profile of novel sponge-associated marine bacteria. J. Ind. Microbiol. Biotechnol. 36:1047-1056.

Dobretsov S, Qian P-Y (2004). The role of epibotic bacteria from the surface of the soft coral Dendronephthya sp. in the inhibition of larval settlement. J. Exp. Mar. Biol. Ecol. 299:35-50.

El-Gendy MMA, HawasUW, Jaspars M (2008). Novel bioactive metabolites from a marine derived bacterium *Nocardia* sp. ALAA 2000. J. Antibiot. 61:379-386.

Hammami I, Rhouma A, Jaouadi B, Rebai A, Nesme X (2009). Optimization and biochemical characterization of a bacteriocin from a newly isolated *Bacillus subtilis*strain 14B for bio-control of *Agrobacterium* sp. Strains. Lett. Appl. Microbiol. 48:253-260.

Jiang P, Li J, Han F, Duan G, Lu X (2011). Antibiofilm Activity of an Exopolysaccharide from Marine Bacterium *Vibrio* sp. QY101. PLoS ONE 6: e18514.

Lili H, Weiliang C,Yang L (2006). Production and Partial Characterization of bacteriocin-like peptides by *Bacillus licheniforms* ZJU123.Microbiol. Res. 161:321-326.

Nithya C, AravindrajaC,PandianSK (2010). *Bacillus pumilus*of Palk Bay origin inhibits quorum-sensing-mediated virulence factors in Gram-negative bacteria. Res. Microbiol. 161:293-304.

Pascal JD, Kareen S, Benoit G,MazzaG (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils.Int.J. Food Microb. 74:101-109.

Qi S-H, Xu Y, Gao J, Qian P-Y, Zhang S (2009). Antibacterial and antilarval compounds from marine bacterium Pseudomonas rhizosphaerae. Annals Microbiol. 59(2):229-233.

Roy S, Rao K, Bhuvaneswari C, Giri A, Mangamoori LN (2010). Phytochemical analysis of Andrographispaniculata extract and its antimicrobial activity. World J. Microbiol. Biotechnol. 26:85–91.

Selvin J, ShanmughapriyaS, R Gandhimathi (2009). Optimization and production of novel antimicrobial agents from sponge associated marine actinomycetes*Nocardiopsisdassonvillei*MAD08. Appl. Microbiol. Biotechnol. 83:435-445.

Sher D, Thompson JW, Kashtan N, Croal L, Chisholm SW (2011). Response of Prochlorococcus ecotypes to co-culture with diverse marine bacteria. The ISME J. 5:1125-1132.

Thenmozhi R, Balaji K, Kumar R, Rao TS, Pandian SK (2011). Characterization of biofilms in different clinical M serotypes of Streptococcus pyogenes. J. Basic Microbiology. 51:196–204.

Thenmozhi R, Nithyanand P, Rathna J, Pandian SK (2009). Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of *Streptococcus pyogenes.* FEMS Immunol. Medical Microbiol. 57:284-294.

Todorov SD, Dicks LMT (2005). *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against gram negative bacteria. Enzyme Microb. Technol. 36:318-326.

Valle JR, Henry DS, Fontaine N, Balestrino T, Latour-Lambert P (2006). Broad-spectrum biofilm inhibition by a secreted bacterial polysaccharide. Proc. Natl. Acad. Sci. USA.103:12558-12563.