Aquat. Living Resour. 28, 99–109 (2015)

DOI: 10.1051/alr/2016004
www.alr-journal.org

Aquatic Living Resources

Influence of low salinity stress on virulence and biofilm formation potential in *Vibrio alginolyticus*, isolated from the Gulf of Khambhat, Gujarat India

Paras DAYMA[1,a], Ishan H. RAVAL[2,a], Nidhi JOSHI[1], Neha P. PATEL[2], Soumya HALDAR[2,b], and Kalpana H. MODY[2]

[1] Marine Biotechnology and Ecology Division, CSIR Central Salt and Marine Chemicals Research Institute, 364002, Bhavnagar, India
[2] Academy of Scientific and Innovative Research (AcSIR), CSIR-CSMCRI, G.B. Marg, 364002, Bhavnagar, India

Received 21 September 2015; Accepted 15 January 2016

Abstract – The Gujarat government has proposed a project to convert a part of the Gulf of Khambhat (GoK), located in western India, into the world's largest man-made freshwater reservoir in the near future (http://www.kalpasar.gujarat.gov.in/mainpage.htm). Anticipating the impact of the change on native bacterial communities, 145 bacteria were isolated initially from different parts of GoK, and further narrowed down to 12 on the basis of diversified geographic distribution in the GoK for further study. All the isolates were able to grow in the complete range of salinity tested (1% to 4%), however an influence of salinity on biofilm formation was observed in only a few isolates. Interestingly, two isolates (91 and 103) identified as *Photobacterium damselae* and *Vibrio alginolyticus* by MIDI and 16sr RNA sequencing were able to produce biofilm only at low salinity conditions (1% to 2%). Along with biofilm formation, isolate 103 showed higher virulence potential in an *Artemia* model only at lower salinity (1%). Therefore, isolate 103 was selected for further characterisation and the image of biofilm developed on a time-scale basis (up to 24 h) for this isolate was studied using a scanning electron microscope (SEM). The change in biofilm surface morphology was studied by atomic-force microscopy (AFM). Live/dead bacterial composition of 24 hour-old matured biofilm also showed that the number of dead bacteria was higher than live bacteria. To the authors' best knowledge, this is the first study showing a change in virulence potential as well as biofilm formation by marine bacteria, exclusively under low salinity stress.

Keywords: Biofilm / Salinity stress / GoK / Marine Vibrio

1 Introduction

The Gujarat government has formulated an ambitious project named Kalpasar to construct a dam across the Gulf of Khambhat (GoK) and thus create a freshwater reservoir by gradually replacing the saline water by inflow of river waters. After completion of the project, it will become the world's largest man-made freshwater reservoir, aiming to supply irrigation water for 1.054 million hectares of land in 39 talukas of 6 districts in the Saurashtra region (http://www.kalpasar.gujarat.gov.in/mainpage.htm).

Anticipating the conversion of part of the GoK area into a freshwater lake, it is assumed that there will be a huge impact on the existing flora and fauna in the gulf. It has been reported that the GoK has high bacterial diversity (Dave and Desai 2006). Recently, Shihora (2013) identified a series of halotolerant bacteria from the GoK. As part of the environmental impact assessment (EIA) of the proposed Kalpasar project, our group isolated and identified a series of marine and coastal bacteria from both sediment and water samples at 11 selected sites in the GoK.

The effects of environmental factors, i.e. changes in temperature and salinity, on the physiology of marine invertebrates have been documented for a variety of mollusks, annelids, and crustaceans (Bayne 1975; Resgalla Jr. C. et al. 2007; Brierley and Kingsford 2009), and several species of echinoderms (Roller and Stickle 1993; Coteur et al. 2002, 2005). Pontarp et al. (2013) have shown that the phylogenetic structure of a bacterial community varies with changes in salinity and dissolved organic carbon. It was also reported that fertilization and early development of the polychaete *Nereis virens* is rarely successful at salinity below 22 practical salinity units (psu) (Allen and Pechenik 2010). As microorganisms act as the base of any ecosystem, understanding the impact of salinity transition over time is very important.

In general, microbes do not live as a pure culture of dispersed single cells but rather accumulate at interfaces to form polymicrobial aggregates, such as biofilms. Although the chemical and biological processes of biofilm formation are poorly understood, it is assumed that at the initial stage, organic and inorganic particles are attached to the surface,

[a] Both the authors contributed equally.
[b] Corresponding author: shaldar@csmcri.org

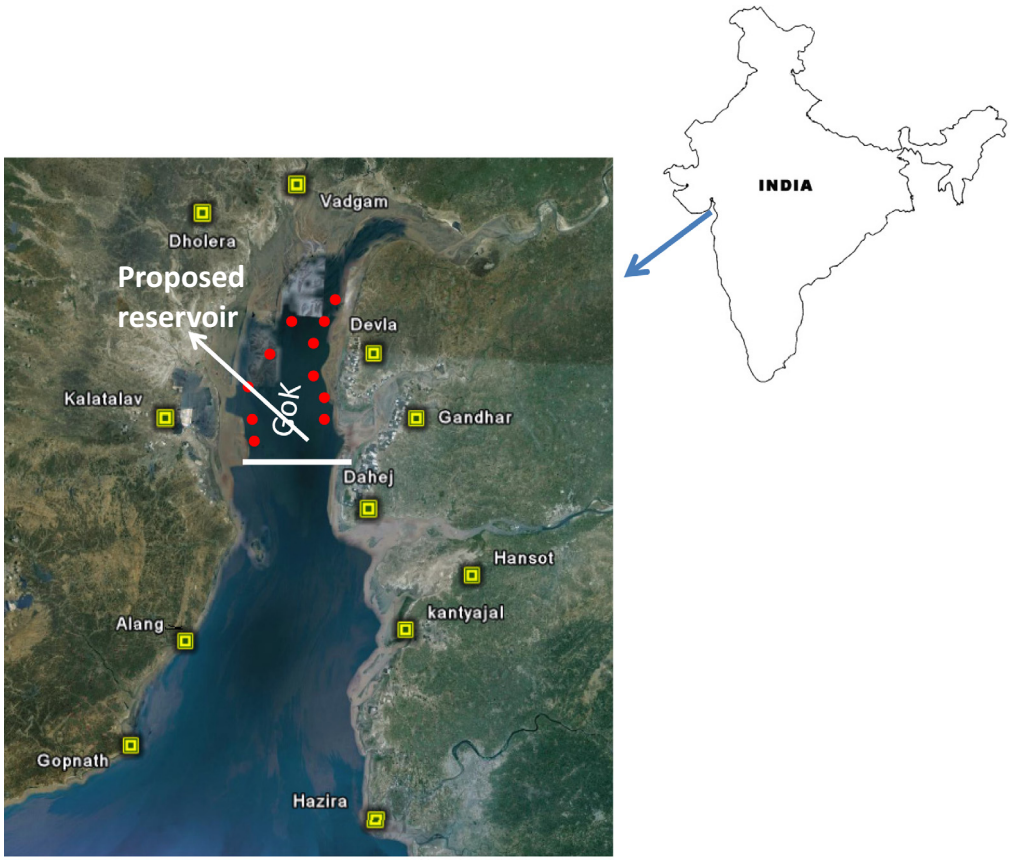


Fig. 1. Map of the GoK showing the 12 locations from where experimental strains were collected.

followed by attachment of pioneer microorganisms. Subsequently, members of primary colonisers grow, reproduce and develop a matured matrix of biofilm (Zobell 1943; Kjelleberg 1982; Garrett et al. 2008; Vu et al. 2009).

Bacterial biofilm formation generally serves as a shield against different environmental and ecological stresses, such as changes in temperature, pH, salinity, etc. (Davey and O'Toole 2000; Di Bonaventura et al. 2007). Keersmaecker et al. (2007) have proven that clinically important probiotics like *Lactobacillus rhamnosus* developed biofilm in an environment with high pH, and in the presence of bile, mucins, and non-digestible polysaccharides. Rao (2010) found that the nature of marine biofilm near a nuclear power plant varied with increases in temperature. Kamjumphol et al. (2013) studied the impact of different environmental factors like salinity, temperature, and presence of iron and manganese on biofilm formation of *Burkholderia pseudomallei*, a soil bacterium. They observed that high osmolarity (NaCl), high iron content, and low temperature promote biofilm formation. Similar findings have also been suggested by other researchers (O'Toole and Kolter 1998; Romling et al. 1998; Singh et al. 2002; White-Ziegler et al. 2008).

As stated earlier, there are many reports describing development of biofilm due to increases in salinity stress (Moldoveanu 2012; Zhang et al. 2011; Mogilnaya et al. 2005; Knobloch et al. 2001). However, to the best of our knowledge, no report describes formation of biofilm by marine bacteria only at low salinity. Therefore, in the present study an isolated and identified *V. alginolyticus* strain was selected on the basis of its pathogenic potential and attempts were made to characterise biofilm formation and changes in protein profiles in low salinity, both in laboratory conditions (culture broth) and in the marine microcosm environment with low salinity. *V. alginolyticus* is considered to be an important human pathogen, causing different types of skin infections and septicaemia (Schmidt et al. 1979). It is also responsible for sporadic diarrhoea in humans (Caccamese and Rastegar 1999).

2 Materials and methods

2.1 Isolation and identification of diversified bacteria

Water and sediment samples were collected from both the eastern and western sides of the GoK and inoculated on Zobell marine agar. Out of 145 marine bacterial isolates, 12 were selected for the study based on their diversified geographical distribution (Fig. 1). All the isolates were stored in 80% glycerol at −80 °C in a deep freezer.

2.2 Salinity tolerance tests

For the salinity tolerance study, all the 12 selected isolates were grown in Luria Bertani (LB) broth with 1% to 4% NaCl

Table 1. Growth and biofilm formation potential at different salinity (1 to 4%) by selected bacterial strains isolated from GoK.

Strain ID	Identification based on fatty acid profiling	Range of Salt concentrations in LB broth													
		1% NaCl		1.5% NaCl		2.0% NaCl		2.5% NaCl		3.0% NaCl		3.5% NaCl		4% NaCl	
		Growth	Biofilm	Growth	Biofilm	Growth	Biofilm	Growth	Biofilm	Growth	Biofilm	Growth	Biofilm	Growth	Biofilm
36	*Grimonttia hollisae*	√	++++	√	++++	√	++	√	++	√	++	√	++	√	++
42	*Grimonttia hollisae*	√	++	√	++	√	++	√	++	√	++++	√	++++	√	–
47	*Grimonttia hollisae*	√	++	√	++	√	++	√	++	√	++	√	++	√	++
88	*Vibrio natrigens*	√	–	√	–	√	–	√	–	√	–	√	–	√	–
90	*Vibrio diazotrophicus*	√	++	√	++	√	++	√	++	√	++	√	++	√	++
91	*Photobacterium damselae*	√	++	√	++	√	++	√	++	√	–	√	–	√	–
93	*Aeromonas hydrophila*	√	–	√	–	√	–	√	–	√	–	√	–	√	–
95	*Shigella dysentery*	√	++	√	++	√	++	√	++	√	++	√	++	√	++
97	*Vibrio fluviatis*	√	++++	√	++	√	++	√	++	√	++	√	++	√	++
98	*Vibrio parahaemolyticus*	√	–	√	–	√	–	√	–	√	–	√	–	√	–
103	*V. alginolyticus*	√	++	√	++	√	++	√	–	√	–	√	–	√	–
108	*Pseudomonas aeruginosa*	√	++++	√	++++	√	++++	√	++++	√	++++	√	++	√	++

+++ High biofilm formation, ++ Moderate biofilm formation, – No biofilm formation.

concentration in 50 mL conical flasks at static conditions. Visible physiological changes marked by growth and formation of biofilm were studied for 48 h (Table 1). Based on the biofilm formation property preferably under low salinity (1% to 2% NaCl concentration), isolates 91 and 103 were selected for the detailed study.

2.3 Detailed bacterial identification based on virulence potential

All 12 isolates were identified on the basis of their fatty acid profiling by MIDI. The method was followed as described in our previous study (Patel et al. 2014). On the basis of MIDI, isolate 91 was identified as *Photobacterium damselae*, which is considered to be a fish pathogen (Romalde, 2002) responsible for pasteurellosis (Thyssen et al. 1998), and isolate 103 as *V. alginolyticus*. Subsequently, to reconfirm the identification, isolate 103 was further identified by 16S rRNA gene sequencing (around 1200 bp) using universal forward primers fD1 (50-AGAGTTTGATCCTGGCTCAG-30) and reverse primer rD1(50-ACGGCTACCTTGTTACGACT-30) as mentioned in our previous study (Patel et al. 2014; Haldar et al. 2011a). In detail, a PCR reaction was performed on BIO-RAD, My Cycler, Thermal Cycler with a mixture of 1 of 100-fold diluted DNA template (30–40 ng), 10 pmol of each primer, 2.5 mmol of each deoxyribonucleotide triphosphate, 1 × PCR buffer and 1U of Taq DNA polymerase and adjusted to a final volume of 50mL with sterile Milli-Q water. The cycles were as follows: 1 cycle at 94 °C for 10 min; 35 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min; and 1 final extension at 72 °C for 7 min. PCR products were checked in 1.5% agarose gel and purification was done using QIAquick PCR purification Kit (Qiagen, England). All the purified DNA products were sequenced in both directions using an ABI PRISM 3100 Avant genetic analyser (Applied Biosystems, M/S, Macrogen, South Korea).

2.4 Virulence potential of *P. damselae* and *V. alginolyticus* in the *Artemia* model at different salinity

The virulence potential of both the strains was checked in an *Artemia* model as described in our previous study (Haldar et al., 2011b). Two sets of seawater were prepared with final salinity of 1.5% and 3.5%. The experiment could not be performed below 1.5% salinity, as *Artemia* can only survive from this salinity upwards. *Artemia* cysts were hatched in gnotobiotic conditions and acclimatised in both the above salinities. Broth culture of both *P. damselae* and *V. alginolyticus* were prepared and inoculated in one set (n = 3) of tubes with 30 mL of seawater with required salinity to yield a final bacterial concentration of approx. 10^7 CFU/mL. Subsequently, 30 newly

hatched acclimatised *Artemia* nauplii were added to each tube. Control was maintained with only *Artemia* nauplii (30). All tubes were kept in shaking conditions (60 rpm) for 24 h at room temperature, and the experiments were carried out in aseptic conditions. Mortality was checked after 24 h. A set of tubes were maintained with only sterile seawater to check for any contamination to the experimental setup (experimental control). On the basis of *Artemia* mortality, isolate 103 was characterised further.

2.5 Effect of different salinity on crude protein profiling of isolate 103

Isolate 103 was grown at both 4% and 1% salinity overnight. Subsequently, bacteria were harvested at 6000 rpm for 10 min at 4 °C. Both precipitate (PT) and supernatant (S) portions were used for further crude protein extraction. PT was sonicated at 40 Hz frequency at 30 s pulse, with a total of 5 pulses. Crude protein was precipitated with ammonium sulphate (40%) from both fractions (PT and sonicated S) and dialysed subsequently with dialysed membrane with pore size 12000 kD (Sigma). Dialysed samples from both PT and S fractions were loaded to 10% SDS gel with a 10 kD marker (Invitrogen).

2.6 Time-scale recovery of low salinity biofilm

For the time-scale study, 10 μL of freshly revived pure culture of isolate 103 was inoculated in forty 100 mL conical flasks containing 50 mL of LB broth (Hi Media, India) supplemented with 1% NaCl concentration. At every 6 h-interval, up to 24 h, biofilms from the top layer of the 10 flasks were harvested, pooled and lyophilised (crude extracts).

Extraction of colloidal and capsular exo-polymers

Both colloidal and capsular exo-polymers were extracted from lyophilised biofilm as described by Aguilera et al. (2008) with slight modification. In brief, 1.5 g of lyophilised dry biofilm was mixed with 5 mL distilled water and kept on a magnetic stirrer for 20 min. Subsequently, the sample was centrifuged at 4000 g for 15 min and the supernatant was collected as a colloidal fraction (Fraction 1). The pellet was mixed with 5 mL of 10 mM EDTA, incubated for 3 h at room temperature (Staats et al. 1999), centrifuged at 16 000 × g for 20 min and the supernatant (capsular fraction) was collected (Fraction 2) (Fig. 2).

Chemical characterisation of both Fractions 1 and 2

Fractions 1 and 2, obtained from crude biofilms collected at different incubation periods (6, 12, 18 and 24 h), were analysed for total sugar and total protein content using the Anthrone (Ludwig and Goldberg 1956) and Lowry (Lowry et al. 1951) methods, respectively. Carbon, hydrogen, nitrogen and sulphate contents were determined by CHNS analyser (Perkin Elmer, CHNS analyser-2400, USA).

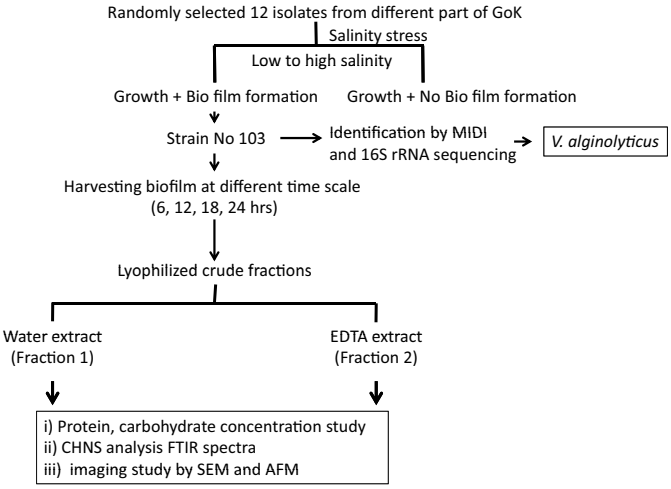


Fig. 2. Flow chart showing how different fractions were extracted from strain 103.

2.7 Chemical characterisation of low salinity biofilm of strain 103 at different time-points (6 h, 12 h, 18 h, 24 h)

FT-IR spectroscopy

The pellet for infrared analysis was prepared by grinding a mixture of 2 mg of lyophylised fraction with 200 mg dry KBr, followed by pressing the mixture into a 16 mm diameter mould. The FT-IR spectrum was recorded in the region of 4000–400 cm^{-1} on a GX FT-IR system (PerkinElmer, USA). FT-IR spectra were taken for samples obtained at regular intervals (6, 12, 18 and 24 h) for all three fractions and major structural groups were detected.

2.8 Microscopy of low salinity biofilm of strain 103 at different time-points (6 h, 12 h, 18 h, 24 h)

Scanning electron microscopy (SEM)

The crude biofilms obtained at different time-points were carefully mounted over a glass cover slip and treated (dehydration) for scanning electron microphotography as described by Yogesh et al. (2010), with slight modification. Step wise dehydration of film was initiated by dipping in 2% glutaraldehyde for 30 min and washing with phosphate buffer of pH 7.2 (two times 5 min each). The mounted samples were transferred gradually to six ascending concentrations of ethanol from 10% to 90% and rectified spirit. The dipping duration was 30 min and it was carried out twice in rectified spirit. The dried samples were then ready for micrographs. SEM allows high-resolution imaging of the cell surface, but requires fixing as described above and then imaging under vacuum may damage the soft biofilm (Dufréne 2002).

Atomic-force microscopy (AFM)

AFM is the only technique that can provide 3D images of the ultra-structure of a surface with molecular resolution,

in real-time under physiological conditions, and with minimal sample preparation. Therefore for the AFM study, crude biofilms produced at different timepoints were directly attached to an acid-washed glass slide and allowed to air dry for imaging (Kavita et al. 2013).

2.9 Live/dead assay of matured biofilm

For the live/dead assay, 24 hour-old biofilm was taken with the help of a cover slip on a clean glass slide and a LIVE/DEAD^{tm} (Invitrogen Ltd.) kit stain was applied to the biofilm, which was kept in the dark for 20 min. Excess stain was then washed off with autoclaved MQ water, two to three times. Biofilm was observed at 100X magnification in an AXIO IMAGER M1 (Carl Zeiss) under green fluorescent protein (GFP) and Rhodamine filters. The combined image reflected both live and dead cells.

3 Results

3.1 Salinity tolerance study

The 12 selected isolates were cultured in LB broth with descending NaCl gradient from 4% to 1%. All the isolates grew well, indicating no effect of different NaCl concentrations on their growth. Three isolates (88, 93 and 98) did not produce biofilm either at low or high NaCl concentrations (Table 1). Interestingly, isolates 91 and 103 developed biofilms exclusively at 1% to 2.5% NaCl concentrations. Remaining isolates developed biofilm irrespective of the NaCl concentration.

3.2 Virulence potential in the *Artemia* model at different salinity

Artemia bath challenge with both isolates 91 and 103 revealed that at 3% salinity, there was no mortality of *Artemia* with both strains. No mortality was recorded in the control either (*Artemia* without any bacteria). However, at 1.5% salinity, 50% mortality of *Artemia* was recorded when they were cultured with isolate 103. No mortality was recorded in the case of isolate 91 in the 1.5% NaCl bath challenge, along with the control. This result suggests that isolate 103, which was identified as *V. alginolyticus* by MIDI, must have developed virulence potential at low salinity along with biofilm formation. Further study was carried out to characterise low salinity biofilm produced by this strain (*V. alginolyticus*).

3.3 Partial 16S rRNA gene sequencing and bacterial identification

A partial 16S rRNA gene sequence of isolate 103 was submitted to DDBJ GenBank (accession number AB933562) and comparison with published sequences in NCBI revealed that this strain had more than 99% homology with *V. alginolyticus* (N26-1). Therefore, identification of this strain was reconfirmed as *V. alginolyticus*.

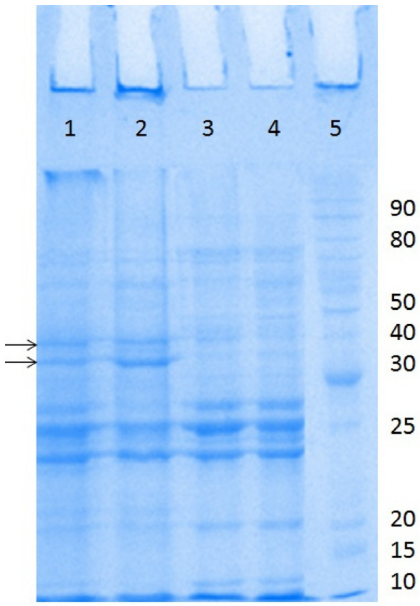


Fig. 3. L1 and L2 denote intracellular and extracellular protein profiles of strain 103 at 1% NaCl concentration. L3 and L4 denote both intra- and extracellular protein profiles at 4% NaCl concentration. L5 denotes protein marker (10 000 D).

Table 2. Protein and carbohydrate concentration in two lyophilized fractions of biofilm developed in different time scales.

Time interval (h)	Lyophilized fraction	% protein	% carbohydrate
6 h	1	29.42	0.86
12 h	1	31.74	0.88
18 h	1	33.65	0.89
24 h	1	40	0.95
6 h	2	26.34	0.92
12 h	2	27.08	0.93
18 h	2	30.7	0.95
24 h	2	32	0.98

3.4 PAGE of crude protein

Polyacrylamide gel electrophoresis (PAGE) of crude extracellular and intracellular proteins extracted from the *V. alginolyticus* strain cultured in 1% and 4% salinity revealed significant differences in band profiling (Fig. 3). Both in the intra- and extracellular fractions, two extra bands were observed when the strain was cultured at 1% salinity.

3.5 Chemical characterisation of Fractions 1 and 2

Analysis of total carbohydrate in the biofilm of strain 103 revealed that total carbohydrate percentage increased from 0.86% to 0.95% for Fraction 1 and from 0.92% to 0.98% for Fraction 2 during its formation from 6 h to 24 h (Table 2). No significant difference in carbohydrate content was observed with time. However, protein content increased significantly from 29% to 40% and 26% to 32% for Fractions 1 and 2, respectively (Table 2).

Table 3. CHNS analysis of two lyophilized fractions of biofilm developed in different time scales.

Time interval (H)	Lyophilized fraction	C%	H%	N%	S%	C:N ratio
6 h	1	25.36	4.66	7.42	0	3.4
12 h	1	35.7	5.889	9.2	0	3.9
18 h	1	40.1	6.148	10.64	0	3.8
24 h	1	37.14	5.71	11.77	0	3.2
6 h	2	25.01	4.65	7.04	0	3.6
12 h	2	33.8	5.944	8.13	0	4.2
18 h	2	32.71	5.43	9.81	0	3.3
24 h	2	36.33	5.951	10.03	0	3.6

C, H, N, S analysis of both Fractions 1 and 2 supported the above data as there was an increasing trend for both C, H and N concentrations in 24 h-old biofilm, as compared to 6-h old biofilm (Table 3). Sulphur was not present in any of these fractions.

Chemical changes over time for three fractions of biofilms were also analysed by FT-IR. There was no significant chemical change in crude fraction with maturation of biofilm. The broad stretch of C-O-C, C-O at 1000–1200 cm^{-1} showed the presence of carbohydrates (Mishra and Jha 2009). Absorption peaks in the range of around 670–516 cm^{-1} correspond to the stretch of alkyl-halides, while those at 2925–29 cm^{-1} and 1414–1253 cm^{-1} revealed the presence of CH_2 and C-H, respectively. This showed asymmetric vibrations (for the CH aliphatic stretching and C-H bending bonds of the -CH_3, -CH_2 and -CH groups), which confirmed the presence of alkanes, and inter and intra-molecular hydrogen bonding. Bands at 1700–1500 cm^{-1} reflected absorption of protein (Jiao et al. 2010; Lal et al. 2010), while peaks at 1640–1654 cm^{-1} (amide I bond and -COO-) exhibited the prominent presence of peptides in all three fractions from the initial stage up to the maturation of biofilm. However, bands at 1547 cm^{-1} corresponding to Amide II (Helm and Naumann 1995) were observed only in 12 h and 18 h fractions of Fractions 1 and 2 (Figs. 4a–4c).

3.6 SEM and AFM analysis

SEM photography of low salinity biofilm of strain 103 at different time-points from 6 h to 24 h revealed a gradual increase in the number of bacterial cells with maturation. Furthermore, at 18 h and 24 h, multiple layers of bacteria along with exopolymeric substances were observed (Fig. 5).

To understand the change in surface structure, AFM was also performed. AFM images revealed a build-up of multiple bacterial layers with gradual maturity of biofilm. This was further schematically represented in Figure 6, where biofilm formation and the process of dispersion are shown.

3.7 Live/dead assay of matured biofilm

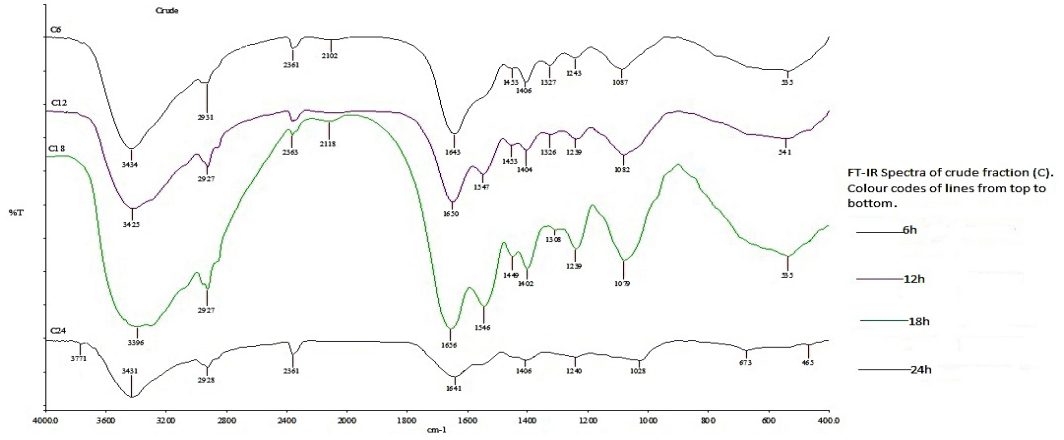
This study showed matured biofilm is mostly composed of dead bacteria (Fig. 7).

4 Discussion

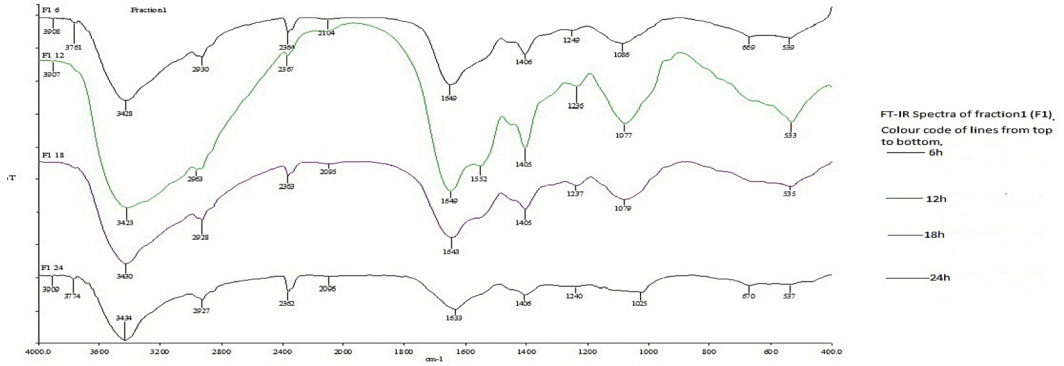
The GoK harbours a diverse bacterial community due to its characteristic geographical location, high tidal amplitude and inflow of anthropogenic/industrial pollutants through river runoff. This study was undertaken to evaluate the impact of low salinity stress on marine bacteria in laboratory conditions, anticipating the conversion of saltwater to freshwater in the proposed reservoir at the GoK.

Two bacteria, which were further identified as *P. damselae* and *V. alginolyticus,* produced biofilm only at low salinity. *Artemia* is considered to be an important animal model for the study of bacterial virulence (Haldar et al. 2011b; Austin et al. 2005). An interesting finding in this study was the development of virulence potential in the *Artemia* model by the *V. alginolyticus* strain only in low salinity. In a previous study of *V. parahaemolyticus* infection in Taiwan Abalone *Haliotis diversicolor supertexta*, the opposite trend was observed. The virulence of *V. parahaemolyticus* in abalone increased with increments in salinity (Cheng et al. 2004).

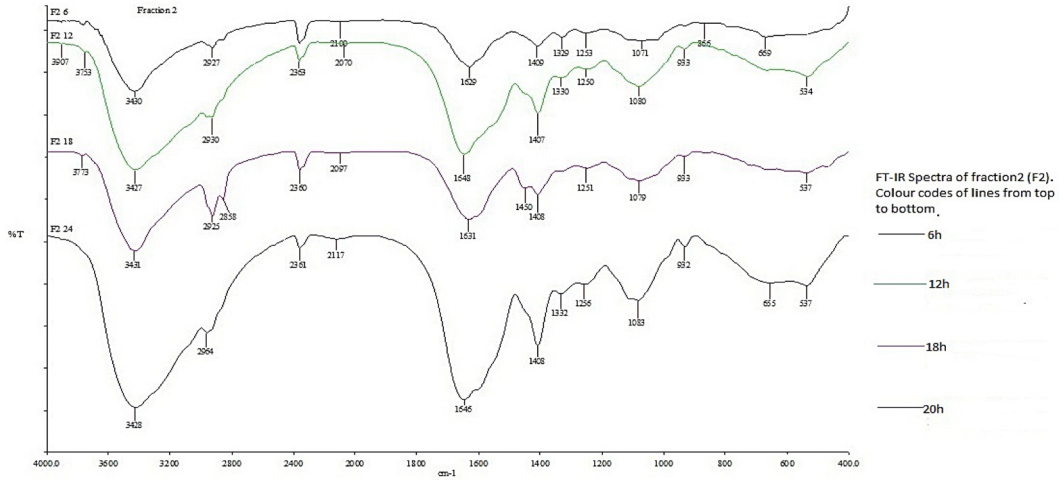
Literature reports showed that the exopolymer of a biofilm generally consists of 40%–95% polysaccharides, 1%–60% proteins, 1%–10% nucleic acids, and 1%–40% lipids (Flemming and Wingender 2002). The composition of exopolymers varies with the composition of microbial consortia and environmental conditions (Kolter and Greenberg 2006). However, in the present study, total carbohydrate content of the biofilm of selected *V. alginolyticus* strains was very low as compared to many other reports (Jiao et al. 2010; Aguilera et al. 2008) (Table 2). It was previously reported that cellular polysaccharides were produced at the early stages of biofilm development, while complex bacterial protein was produced at the later stage of biofilm formation (Bremer and Geesey 1991; Cheung et al. 2000). In the present study, the reason for the high concentration of protein in both the fractions of stressed biofilm of the *V. alginolyticus* strain is yet to be understood. Development of biofilm under a hyposaline environment might be the reason for the high concentration of protein in both the fractions. The SDS PAGE profile also supports this finding. Two extra bands were found in both the extracellular and intracellular fractions from low salinity (1%) samples. Feng et al. (2007) also reported protein expression profiles of *Halobacillus dabanensis* D-8^T under 1%, 10% and 20% salt concentration using two dimensional (2-D) gel electrophoresis. The study concluded that among 133 protein spots detected, 62 showed up-regulation and 26 new protein spots were found under high salinity conditions. However, these changes in protein expression were mainly for survival under highly saline conditions. In the present study, extra bands were also observed in the crude proteins extracted from the *V. alginolyticus* strain cultured at 1% salinity (Fig. 3). This result indicates that this bacterium may be under stress at lower salinity. However, when the concentration of an oxidative stress enzyme such as super oxide dismutase (SOD) was analysed from the bacterial culture in different salinity conditions, it was observed that there was no difference in enzyme production (data not shown). This result indicates that the extra band found in the PAGE profile does not represent the SOD enzyme.



(a)



(b)



(c)

Fig. 4. (a) FTIR analysis of crude fractions at 6 h, 12 h, 18 h and 24 h. (b) FTIR analysis of Fraction 1 at 6 h, 12 h, 18 h and 24 h. (c) FTIR analysis of Fraction 2 at 6 h, 12 h, 18 h and 24 h.

Elemental analysis of C, H, N, S for biofilm is rare. However, there are some reports describing C:N ratio tests in environmental bacterial biofilms. Fukuda et al. (1998) reported C:N ratios of bacteria present in open ocean and coastal areas of 6.8 and 5.9, respectively, which is significantly higher than in the present study. The C:N ratios of Fractions 1 and 2 at different stages of development varied between 3.4 to 4.2 and 3.3 to 3.6, respectively. Moldoveanu (2012) observed that there is no influence on the C:N ratio in developing biofilm due to exposure to different salinity gradients.

In general, amide or its derivatives are potent antioxidants and are responsible for reduction of oxidative stress in organisms (P0061tent No. EP 1877044 A2). However, polyamide level is not associated with high NaCl stress (Kao 1997). In the present study, the amide concentration and ratio with lipid fraction (2963 cm^{-1}) was reported to be high in 12 h and

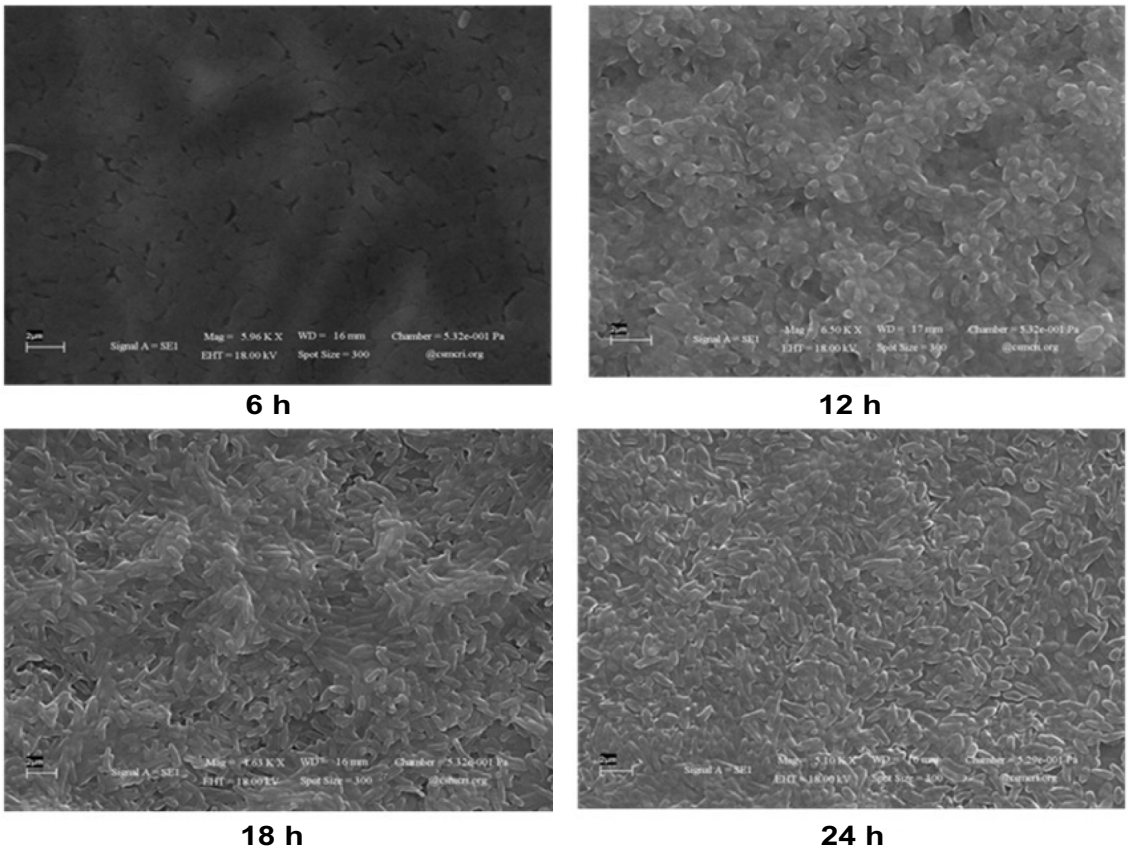


Fig. 5. SEM microphotograph of biofilm of strain 103 at different time-points.

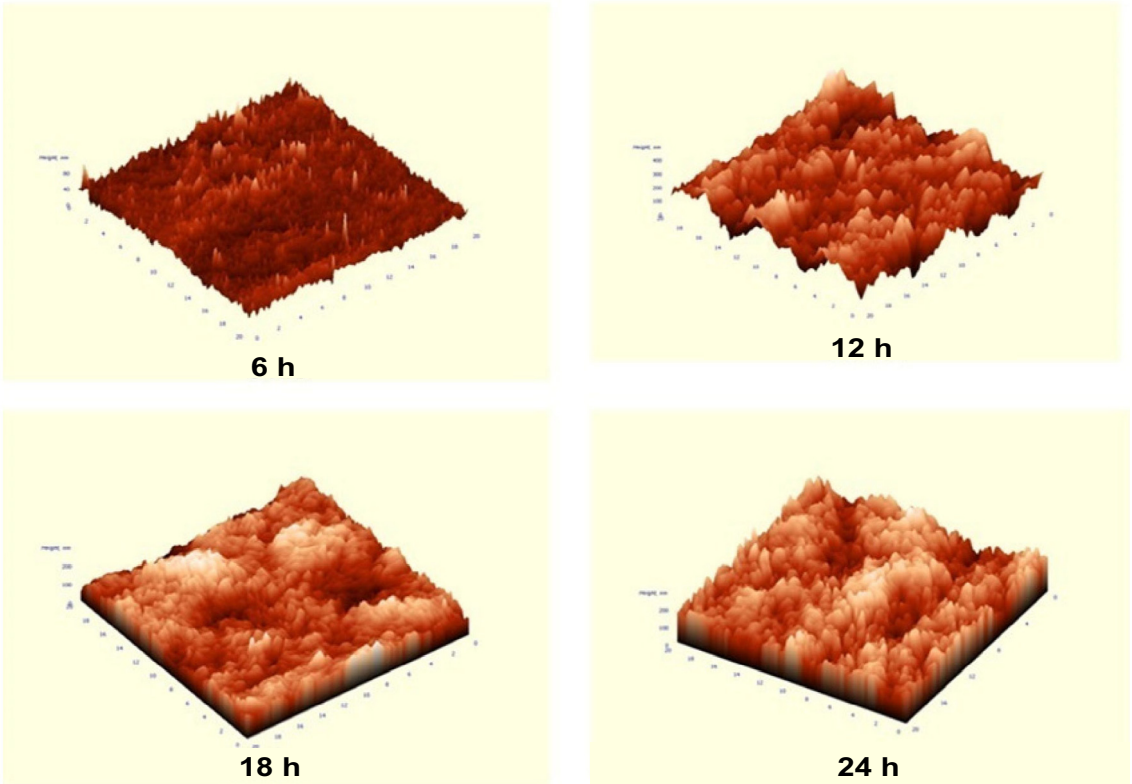


Fig. 6. AFM photograph of biofilm of strain 103 at different time-points.

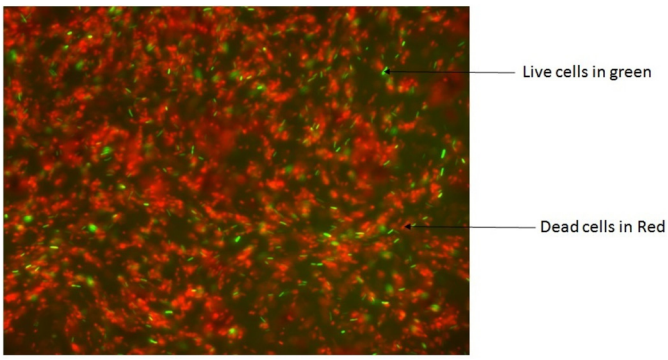


Fig. 7. Live/dead bacterial composition of matured (24 h) biofilm.

18 h fractions. This might be due to low salinity stress in the present study. C-O polysaccharide stretching (Bremer and Geesey 1991) and secondary alcohol were present in all fractions studied in the sugar absorption area. Primary alcohol stretching was observed in crude biofilm and Fraction 2, however absent is all samples of Fraction 1. Similarly, primary alcohol, azides, and halogens were also reported in all the stages in crude extracts of developing biofilm (Figs. 4a–4c). Donlan et al. (2001) performed a detailed real-time analysis of *Streptococcus pneumoniae* biofilm formation and the spectral analysis revealed that amide and polysaccharide bonds were detected in the initial stages of biofilm development and they remained relatively stable until later stages. Similarly, in the present study it was observed that only a nitro group was incorporated with the development of biofilm in later stages in all three extracts.

It is well established that bacteria can protect each other from potentially adverse events such as antibiotic pressure and environmental stress, especially when cells are located within a biofilm (Lanwermeyera et al., 2014). Presence of a high number of dead bacteria in the live/dead assay of matured bacteria proved that the dead bacteria may act as a protective sheet that may help live bacteria to develop more resistance against different antibiotics.

5 Conclusion

It is well recognised that Vibrios are one of the dominant bacterial genera in seawater. After conversion of the GoK to a freshwater reservoir, there may be a considerable impact on the bacterial communities, especially to marine Vibrios. The impact includes development of biofilm formation as reported in the present study in the case of *P. damselae* and *V. alginolyticus*, and an increase in virulence potential of *V. alginolyticus* in the *Artemia* model. A detailed investigation is required to understand the impact of salinity changes in the other marine organisms, as well as over the whole gulf ecosystem.

Acknowledgements. We sincerely thank ADCIF for providing all instruments and facilities. Special thanks are due to Mr. Jayesh Chaudhury (SEM analysis), Dr. Babulal Rebary (AFM analysis), Mr. Vinod Agarwal (FTIR analysis) and Dr. Santlal Jaiswar (for capturing the fluorescent microscopy photograph). Financial support to IR and NP by DST-INSPIRE and CSIR are duly acknowledged. The manuscript has been assigned registration number CSIR-CSMCRI – 074/2015.

References

Allen J.D., Pechenik J.A., 2010, Understanding the Effects of Low Salinity on Fertilization Success and Early Development in the Sand Dollar *Echinarachnius parma.* Biol. Bull. 218, 189–199.

Aguilera A., Souza-Egipsy V., Martín-Úriz P.S., Amils R., 2008, Extraction of extracellular polymeric substances from extreme acidic microbial biofilms. Appl. Microbiol. Biotechnol. 78, 1079–1088.

Austin B., Austin D., Southerland R., Thompson F., Swings J., 2005, Pathogenicity of vibrios to rainbow trout (*Oncothynchus mykiss*, Walbaum) and *Artemia* nauplii. Environ. Microbiol. 7, 1488–1495.

Bayne B.L., 1975, Aspects of physiological conditions inMytilus edulis L.,with special reference to the effects of oxygen tension and salinity. In: Gray, J.S., Christiansen, M.E. (Ed.), Proc. 9th Europ Mar. Biol. Symp. John Wiley and Sons Ltd., Chichester, pp. 331–349.

Bremer P.J., Geesey G.G., 1991, An evaluation of biofilm development utilizing non-destructive attenuated total reflectance Fourier transform infrared spectroscopy. Biofouling 3, 89–100.

Brierley A.S., Kingsford K.J., 2009, Impacts of Climate Change on Marine Organisms and Ecosystems. Curr. Biol. 19, R602–R614.

Caccamese S.M., Rastegar D.A., 1999, Chronic Diarrhea Associated with Vibrio alginolyticus in an Immunocompromised Patient. Clin. Infect. Dis., 29, 946–7.

Cheng W., Juang F.M., Chen J.C., 2004, The immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus* at different salinity levels. Fish Shellfish Immunol.16, 295–306.

Cheung H.Y., Sun S.Q., Ching B., Sreedhar W.M., Tanner P.A., 2000, Alterations in extracellular substances during the biofilm development of Pseudomonas aeruginosa on aluminium plates. J. Appl. Microbiol. 89, 100–106.

Coteur G., Warnau M., Jangoux M., Dubois P, 2002, Reactive oxygen species (ROS) production by amoebocytes of Asterias rubens (Echinodermata). Fish. Shelfish. Immunol. 12, 187–200.

Coteur G., Gillan D., Pernet Ph., Dubois Ph., 2005, Alteration of cellular immune responses in the seastar Asterias rubens following dietary exposure to cadmium. Aquat. Toxicol., 73, 418–421.

Dave S.R., Desai H.B., 2006, Microbial diversity at marine salterns near Bhavnagar, Gujarat, India. Curr. Sci. 90, 497–500.

Davey M.E., O'Toole G.A., 2000, Microbial biofilms: from ecology to molecular genetics. Microboil. Mol. Biol. Rev. 64, 847–67.

Di Bonaventura G., Stepanoviæ S., Picciani C., Pompilio A., Piccolomini R., 2007, Effect of environmental factors on biofilm formation by clinical Stenotrophomonas maltophilia isolates. Folia Microbiol. (Praha). 52, 86–90.

Donlan R.M., Murga R., Bell M., Toscano C.M., Carr J.H., Novicki T.J., Zuckerman C., Corey L.C., Miller J.M., 2001, Protocol for detection of biofilms on needleless connectors attached to central venous catheters. J. Clin. Microbiol. 39, 750–753.

Dufréne Y.F., 2002, Mini-review. Atomic force microscopy, a powerful tool in microbiology. J. Bacteriol. 184, 5205–5213.

Feng D.Q., Yang L.F., Lu W.D., Yang S., 2007, Analysis of Protein Expression Profiles of Halobacillus dabanensis D-8T Under Optimal and High Salinity Conditions. Curr. Microbiol. 54, 20–26.

Flemming H.C., Wingender J., 2002, Extracellular Polymeric Substances (EPS): Structural, Ecological and Technical aspects, In: Bitton G, Eds. Encyclopedia of environmental microbiology, John Wiley & Sons, New York, pp. 1223–1231.

Fukuda R, Ogawa H., Nagata T., Koike I., 1998, Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. Appl. Environ. Microbiol. 64, 3352–3358.

Garrett T.R., Bhakoo M., Zhang Z., 2008, Bacterial adhesion and biofilms on surfaces. Prog. Natural Sci. 18, 1049–1056.

Guerin J.L., Stickle W.B., 1992, Effects of salinity gradients on the tolerance and bioenergetics of juvenile blue crabs (*Callinectes sapidus*) from waters of different environmental salinities. Mar. Biol. 114, 391–396.

Haldar S., Mody K.H., Jha B., 2011a, Abundance, diversity and antibiotics resistance pattern of *Vibrio* spp. in coral ecosystem of Kurusadai island, J. Basic Microbiol. 51, 153–162.

Haldar S., Chatterjee S., Sugimoto N., Das S., Chowdhury N., Hinenoya A., Asakura M., Yamasaki S., 2011b, Identification of *Vibrio campbellii* isolated from diseased farm-shrimps from south India and establishment of its pathogenic potential in an *Artemia* model. Microbiology-SGM 157, 179–188.

Helm D., Naumann D., 1995, Identification of some bacterial cell components by FTIR spectroscopy. FEMS Microbiology Lett. 126, 75–80.

Jiao Y., Cody G.D., Harding A.K., Wilmes P., Schrenk M., Wheeler K.E., Banfield J.F., Thelen M.P., 2010, Characterization of extracellular polymeric substances from acidophilic microbial biofilms. Appl Environ Microbiol 76(9), 2916–2922.

Kamjumphol W., Chareonsudjai S., Chareonsudjai P., Wongratanacheewin S., Taweechaisupapong S., 2013, Environmental factors affecting *burkholderia pseudomallei* biofilm formation. Southeast Asian J. Trop. Med. Public Health 44, 72–81.

Kao C.H., 1997, Physiological significance of stress-induced changes in polyamines in plants. Bot. Bull. Acad. Sin. 38, 141–144.

Kavita K., Mishra A., Jha B., 2013, Extracellular polymeric substances from two biofilm forming Vibrio species: Characterization and applications. Carbohydr. Polym. 94, 882–88.

Kjelleberg S., Lagercratz C., Marshall K.C., 1982, The effect of interfaces on small starved marine bacteria. Appl. Environ. Microbiol. 43, 1166–72.

Knobloch J.K., Bartscht K., Sabottke A., Rohde H., Feucht H.H., Mack D., 2001, Biofilm formation by Staphylococcus epidermidis depends on functional RsbU, an activator of the sigB operon: differential activation mechanisms due to ethanol and salt stress. J. Bacteriol. 183, 2624–33.

Kolter R., Greenberg E.P., 2006, Microbial sciences: The superficial life of microbes. Nature 441, 300–302.

Lal P., Sharma D., Pruthi P., Pruthi V., 2010, Exopolysaccharide analysis of biofilm- forming Candida albicans. J. Appl. Microbiol. 109, 128–136.

Lanwermeyera S.K., Xia C., Jakubovicsb N.S., Rickardc A.H., 2014, Microbial coaggregation: ubiquity and implications for biofilm development. Biofauling 30, 1235–1251.

Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J., 1951, Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Ludwig T.G., Goldberg H.J.V., 1956, The Anthrone Method for the Determination of carbohydrates in Foods and in Oral Rinsing. J. Dent. Res. 35, 90–94.

Mishra A., Jha B., 2009, Microbial Exopolysaccharides, In: Rosenberg E. et al., (Eds.), The Procaryotes-Applied Bacteriology and Biotechnology, Springer-Verlog, Berlin, Heidelberg, pp. 179–192.

Mogilnaya O.A., Lobova T.I., Kargatova T.V., Popova L.Y.U., 2005, Biofilm formation by bacterial associations under various salinities and copper ion stress. Biofouling 21, 247–255.

Moldoveanu A.M., 2012, The Influence of Mytilus Extract on Biofilm Cells Attachment. Annals of the Romanian Society for Cell Biology, 17(1), 111.

O'Toole GA, Kolter R., 1998, Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. 28, 449–61.

Patel M., Baxi K., Dayma P., Upadhyay D., Parmar N., Kundu S., Haldar S., Mody K..H., Jha B., 2014, Assessment of Ground Water Quality with Respect to Bacteriological Contamination in Bhavnagar, Gujarat, India. Clean – Soil Air Water 42, 1351–1362.

Patent No. EP 1877044 A2. N-acetylcysteine amide (nac amide) for the treatment of diseases and conditions associated with oxidative stress.

Pontarp M., Sjöstedt J., Per Lundberg P., 2013, Experimentally induced habitat filtering in marine bacterial communities. Mar. Ecol. Prog. Ser. 477, 77–86.

Rao T.S., 2010, Comparative effect of temperature on biofilm formation in natural and modified marine environment. Aquat. Ecol. 44, 463–478.

Resgalla Jr. C., Brasil Ed.S, Salomão L.C., 2007, The Effect of Temperature and Salinity on the Physiological Rates of the Mussel Perna perna (Linnaeus 1758). Braz. Arch. Biol. Technol. 50, 543–556.

Romalde J.L., 2002, Photobacterium damselae subsp. piscicida: an integrated view of a bacterial fish pathogen. Int. Microbiol. 5, 3–9.

Romling U., Sierralta W.D., Eriksson K., Normark S., 1998, Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the agfD promoter. Mol. Microbiol. 28, 249–64.

Lebeer S., Verhoeven T.L.A., Vélez M.P., Vanderleyden J., Keersmaecker S.C.J.D., 2007, Impact of Environmental and Genetic Factors on Biofilm Formation by the Probiotic Strain *Lactobacillus rhamnosus* GG. Appl. Environ. Microbiol. 73, 6768–6775.

Schmidt U, Chmel H, Cobbs C., 1979, Vibrio alginolyticus Infections in Humans. J Clin. Microbiol. 10, 666–668.

Shihora N. A., 2013, Isolation and characterizations of halotolerant bacteria and identification by FAME analysis. J. Appl Res. 3, 51-53.

Singh P.K., Parsek M.R., Greenberg E.P., Welsh M.J., 2002, A component of innate immunity prevents bacterial biofilm development. Nature 417, 552–555.

Staats N., Winder B.D., Stal L.J., Mur L.R., 1999, Isolation and characterization of extracellular polysaccharides from the epipelic diatoms *Cylindrotheca closterium* and *Navilcula salinarum*. Eur. J. Phycol. 34, 161–169.

Thyssen A., Grisez L., van Houdt R., Ollevier F., 1998, Phenotypic characterization of the marine pathogen Photobacterium damselae subsp. piscicida. Int. J. Syst. Bacteriol. 4, 1145−51.

Vu B., Chen M., Crawford R.J., Ivanova E.P., 2009, Bacterial Extracellular Polysaccharides Involved in Biofilm Formation. Molecules 14, 2535−2554.

White-Ziegler C.A., Um S., Perez N.M., Berns A.L., Malhowski A.J., Young S., 2008, Low temperature (23 °C) increases expression of biofilm-, cold-shock- and RpoS-dependent genes in *Escherichia coli* K−12. Microbiology 154, 148−66.

Yogesh, Haldar S., Paul P, Bhattacharya A., 2010, Polysulfone-Azo Composite Membrane: New Preparative Approach, Importance in Bactericidal and Biofilm Inhibition Activities. J. Appl. Polymer Sci. 115, 3710−3715.

Zhang Z., Chen S.H., Wang S.M., Luo H.Y., 2011, Characterization of extracellular polymeric substances from biofilm in the process of starting-up a partial nitrification process under salt stress. Appl. Microbiol. Biotechnol. 89, 1563−1571.

Zobell C.E., 1943, The effect of solid surfaces on bacterial activity. J. Bacteriol. 46, 39−56.