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RESEARCH ARTICLE

Isolation and characterization of marine biofilm forming bacteria from a ship's hull

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BACKGROUND: Diverse aquatic microorganisms are capable of colonizing living and non-living surfaces leading to the formation of biofilms. Commonly visualized as a slimy layer, these biofilms are filled with hundreds of other microorganisms compared to free living planktonic cells. Microbial surface colonization and surface-associated metabolic activities also exert several macroscale deleterious effects, including biofouling, biocorrosion and the persistence and transmission of harmful or pathogenic microorganisms and virulence determinants. The present study deals with the isolation and screening of marine bacteria for biofilm formation. The screened isolates were characterized and identified as *Psychrobacter celer*, *Psychrobacter alimentarius* and *Kocuria rhizophila* by 16S rRNA sequencing.
METHODS: Biofilm forming bacteria were isolated by spread plate technique and subjected to screening by microtiter plate assay. The potent biofilm formers were identified by molecular characterization using 16S rRNA gene sequencing.
RESULTS: Twelve bacterial isolates were obtained by pour plate technique and subjected to biofilm assay. Among the 12 isolates three isolates which showed maximum biofilm formation were subjected to molecular characterizationby 16S rRNA gene sequencing method. The isolates were identified as *Psychrobacter celer*, *Psychrobacter alimentarius* and *Kocuria rhizophila*. The EPS produced by the three biofilm forming bacteria was extracted and the protein and carbohydrate content determined.
CONCLUSION: Among the isolates screened, isolate 8 (*Kocuria rhizophila*) produced maximum protein and carbohydrate which was also in accordance with the results of microtiter plate assay.

Keywords biofilm, MTP assay, extracellular polymeric substances, 16S rRNA gene sequencing, *Psychrobacter*, *Kocuria*

Introduction

In marine environment bacteria commonly exist in a self-produced polymeric matrix adherent to an inert or living surface. Initial surface colonization and subsequent biofilm formation and development provide numerous benefits to these organisms and support critical ecological and biogeochemical functions in the changing marine environment. Such surface association seems to be an ancient, universal, and fundamental survival mechanism that offers microorganisms with critical benefits, including greater access to nutritional resources, enhanced organism communications, and greater environmental stability. These features are of particular significance in natural aquatic environments where the availability of nutrients is often growth limiting and ambient conditions are highly dynamic and sometimes harmful (Costerton et al., 1995; Hall-Stoodley et al., 2004; Trevors, 2011). Charged and hydrophobic materials have a tendency to settle on submerged surfaces, and biogenic particles like phytoplankton debris, zooplankton fecal pellets, and marine snow rich in organic matter provide easy access to various nutrients in otherwise intense nutrient-limited environments (Costerton et al., 1995; Beveridge et al., 1997; Dang and Lovell, 2000; Davey and O'Toole, 2000; Watnick and Kolter, 2000; Hall-Stoodley et al., 2004). The settlement of biofilm on submerged surfaces can be distributed into three main phases: a) an initiation phase in the course of which chemical adsorption of organic chemicals may occur on the material surface and the first adhesion of few bacteria b) a second phase during which there is an increase in the number of adhesive microorganisms c) a third phase, a pseudo-

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stationary phase, during which an equilibrium exist between bacterial development and detachment and thus the biofilm thickness is more or less constant (Feron and Dupont, 1995). Microbial surface colonization and surface-associated metabolic activities also exert several macroscale deleterious effects, including biofouling (Steinberg et al., 2002; Salta et al., 2013), biocorrosion (Newman and Banfield, 2002; Beech and Sunner, 2004; Dang et al., 2011), and the persistence and transmission of harmful or pathogenic microorganisms and virulence determinants (Decho, 2000; Cottingham et al., 2003; Huq et al., 2008). Microorganisms belonging to different groups are known to form biofilms. Among bacteria several species of *Pseudomonas, Aerobacter aerogens, Bacillus alvei* and *Vibrio alginolyticus* have been reported in several studies (Sonak and Bhosle, 1995; Chaudhary et al., 1997; Sakurai and Yoshikawa, 2012). In the present study 12 bacteria were isolated from marine biofilms collected from ship hull. Further these bacterial isolates were screened for biofilm forming ability and three isolates exhibiting maximum biofilm forming ability were identified by 16S rRNA gene sequencing.

Materials and methods

Collection of sample

Biofilm samples were scrapped and collected from the hull of boat anchored at Pichavaram beach (11.4226° N latitude and 79.7748° E longitude respectively), Parangepettai, Chidambaram, Tamil Nadu in sterile centrifuge tubes and aseptically transferred to the laboratory and stored in deep freezer for isolation.

Isolation of Biofilm Forming Bacteria

Biofilm samples were serially diluted and plated by spread plate technique on Zobell Marine Agar (Himedia). Individual colonies were purified by streaking and the pure colonies were screened for their biofilm forming ability using microtiter plate (MTP) assay.

Screening of biofilm forming bacteria

Isolates were screened for their ability to form biofilm by microtiter plate assay (Jordjevic et al., 2002). Overnight grown broth cultures of the isolates (cultured in nutrient agar) were transferred to sterile polystyrene 96 well microtiter plate along with the control (uninoculated broth) and the plate was incubated for 3 days at room temperature. After incubation, the contents of each well were gently removed by slightly tapping the plates, and the wells were washed with phosphate buffer saline (PBS at pH7.4) to remove the planktonic cells. The plates were then stained with 0.1% (w/v) crystal violet solution. Excess stain was washed off thoroughly with 70% ethanol and plates were kept for drying. Optical density (OD) of the wells was determined at 570 nm with a Micro-Plate reader MR 960, OD values $\geqslant 0.01$ were considered as an index of attachment to surface and biofilm formation. The experiment was performed in triplicate and the mean of OD value is presented.

Biochemical characterization

The isolates exhibiting maximum biofilm formation were subjected to morphological and biochemical characterization using standard microbiological approaches and standard biochemical tests. The isolates were further subjected to molecular characterization by 16S rRNA gene sequencing.

Identification of bacterial isolates by 16S rRNA gene sequencing

Molecular identification of isolates 3, isolate 4 and isolate 8 were carried out by 16S rRNA gene sequencing. Genomic DNA was extracted, and amplified by using 8F-(5′ AGAGTTTGATCCTGGCTCAG3′) with 20 base pairs, and 1541R – (5′AAGGAGGTGATCCAGCCGCA3′) with 20 base pairs. Polymerase chain reaction was performed by adding 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on each template using 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were suspended in distilled water and subjected to electrophoresis in an ABI 3730 xl sequencer (Applied Biosystems).

Biofilm architecture by Scanning Electron Microscopy

Sample preparation for scanning electron microscopic (SEM) studies was performed as follows. The biofilms grown on coverslips were fixed for 1 h in a solution containing 2.5% glutaraldehyde. Coverslips were washed in 0.1 M sodium acetate buffer (pH 7.3) and dehydrated in a graded series of ethanol 50%, 75%, 85%, 95%, 99% and final immersion in 100%. Subsequently, coverslips were subjected to critical point drying with CO_2.

Characterization of extracellular polymeric substances from biofilm

The three selected isolates namely isolate 3, isolate 4 and isolate 8 were allowed to form biofilm on glass beads for EPS extraction and characterization. Overnight grown cultures of

the isolates were diluted and 20 ml of the culture broth transferred to a flask containing sterilized glass beads of 2 mm diameter. After incubation of the flasks at 37 C for 72 h, the glass beads were gently washed with PBS in order to remove the planktonic cells and then gently vortexed to disintegrate the biofilm. EPS was extracted by thawing the samples on ice and centrifuging at 15000 g for 20 min. The resultant biofilm pellets were resuspended in 30 ml of 0.2 M cold sulfuric acid and the matrix was homogenized. The cell suspension thus obtained was kept at 4°C for 3 h and centrifuged at 15000 g for 20 min. The supernatant containing total EPS was subjected to chemical analysis and the total carbohydrate and protein were quantified using anthrone method and Lowry's method respectively.

Results and discussion

Isolation and screening of biofilm forming bacteria

Isolation of biofilm forming bacteria on the Zobell Marine agar medium by spread plate technique yielded the bacterial growth averaging 4.32×10^4 CFU g^{-1}. A total of 12 bacterial isolates were obtained and subjected to screening for the biofilm formation by microtiter plate assay. After the primary screening by microtiter plate assay, five isolates (isolate 3, 4, 7, 8 and 9) were found positive for biofilm formation (Fig. 1). Of these, three isolates (isolate 3, isolate 4, and isolate 8) showing maximum biofilm formation was chosen for further study (Fig. 2). Though OD values of all the three isolates were almost similar, isolate 8 could be considered as the most potent biofilm forming isolate. Microtiter plate has the leverage of enabling researcher to promptly analyze the adhesion of various bacterial strains (Anwar et al., 1992; An and Friedma, 2000). Based on the optical density and the mean value, the adherent ability of the bacteria can be determined and grouped as adherent, non- adherent, strong or weak adherent or even moderate adherent biofilm forming bacteria respectively (Xu et al., 2005).

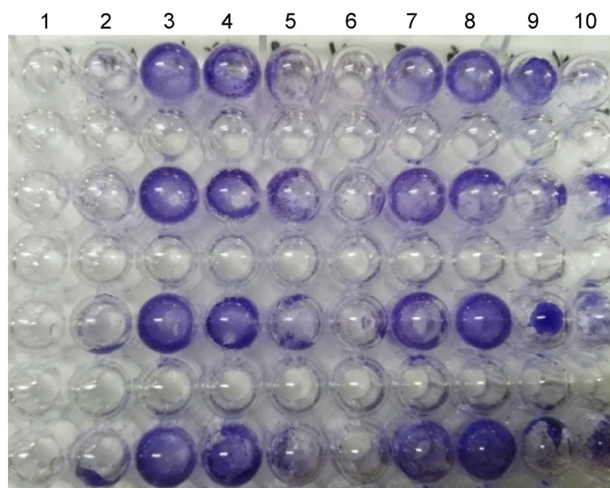

Figure 1 Microtiter plate assay of Isolate 3, Isolate 4 and Isolate 8.

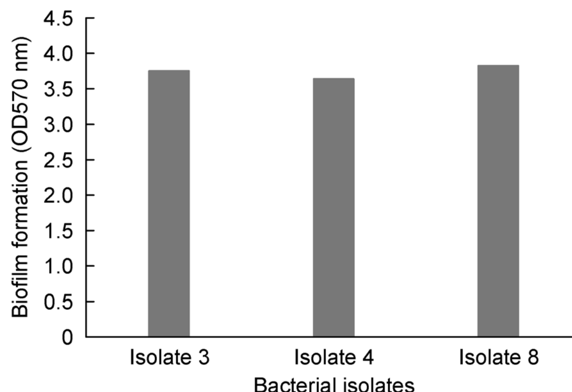

Figure 2 Screening of biofilm forming bacteria by Micro Titer Plate assay.

Table 1 Morphological and biochemical characterization of biofilm forming bacteria

Characteristics	Isolate 3	Isolate 4	Isolate 8
Cell shape	Cocci	Cocci	Cocci
Gram stain	(−) ve	(−) ve	(+) ve
Gelatin	(−) ve	(−) ve	(−) ve
Urease	(−) ve	(−) ve	(−) ve
Nitrate reduction	(−) ve	(−) ve	(−) ve

Biochemical characterization

After testing the biofilm forming ability the isolates were subjected to biochemical characterization. Isolate 3 and isolate 4 were found to be gram negative and isolate 8 was found to be gram positive (Table 1). The cell shape was found to be cocci in all the cases. Isolate 3, Isolate 4 and isolate 8 was found to be negative for gelatin test, urease and nitrate reduction test as well. A discrete cell within a multispecies biofilm usually lives in a unique microniche where nutrients are provided by adjacent cells and by diffusion, where products are removed by the same manner, and where competitors may be kept at a distance by diffusion barriers (Costerton et al., 1994).

Identification of Biofilm forming bacteria by 16S rRNA gene sequencing

Based on 16S rRNA gene sequencing the three biofilm forming isolates were identified as *Psychrobacter celer, Psychrobacter alimentarius and Koucuria rhizophila*, and their sequences were deposited in GenBank (NCBI) under the accession numbers MG262562. 1, MG263509.1 and MG203898.1 respectively. The phylogenetic trees are shown in Fig. 3A, B and C. Members of the genus *Psychrobacter* are reported to be found in saline habitat and

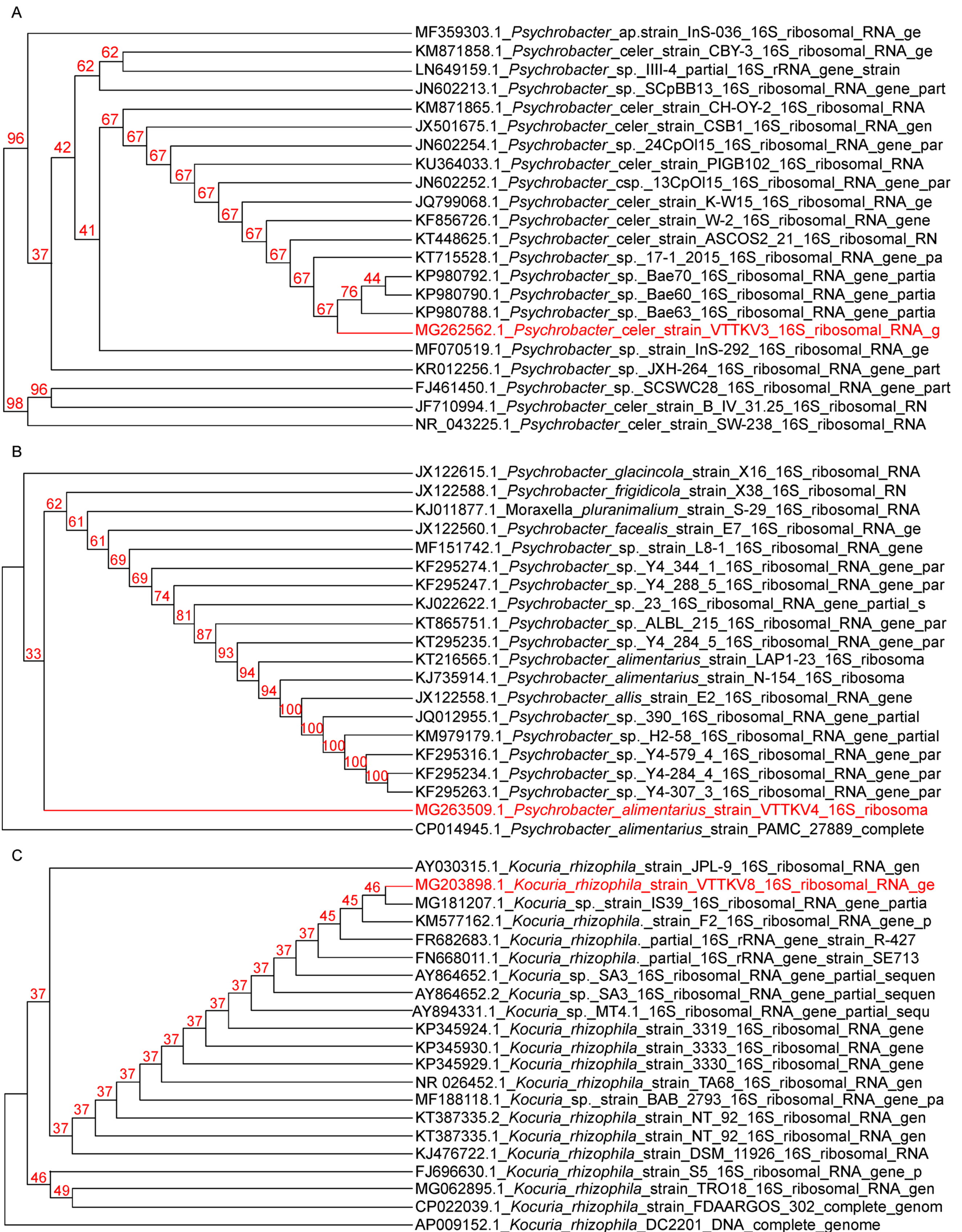

Figure 3 Phylogenetic analysis of biofilm forming bacteria. (A) *Psychrobacter celer,* (B) *Psychrobacter alimentarius,* (C) *Koucuria rhizophila*.

belong to the family *Moraxellaceae* and the class Gammaproteobacteria. *Kocuria* is a genus of Gram positive bacteria in the phylum Actinobacteria and belongs to the family *Micrococcaceae*. Several biofilm forming bacteria have been reported in different environments. *Pseudomonas fluorescens* (Sillankorva et al., 2008), *Vibrio alginolyticus* (Sonak and Bhosle, 1995) are some of the reported biofilm forming bacteria. Most of the marine bacteria from the phylogenetic group of *Gammaproteobacteria* are aerobic or facultative anaerobic.

It is quite evident from the reported literature that biofilms from marine environment harbor diverse group of marine bacteria. Marine bacterial biofilm communities have been explored in several studies and found to contain bacteria with diversified physiology and tolerance capabilities. Members of different groups such as Gram-negative bacteria (Enterobacteriaceae), Gram-positive bacteria (Bacillaceae), rod shaped bacteria (*Bacillus* sp.), coccoid bacteria (*Micrococcus* sp.), halophilic bacteria (*Halomonas aquamarina*) and halotolerant bacteria (*Exiguobacterium* sp.) have been reported to exist as components of biofilm (Priest, 1993; Sass et al., 2001; Yang et al., 2001; Pitt et al., 2007; Aiassa et al., 2010).

Biofilm architecture by scanning electron microscopy (SEM)

SEM images of biofilms formed by *P. celer, P. alimentarius* and *K. rhizophila* are in accordance with reported studies (Li et al., 2002; Pandit et al., 2011). The biofilms comprised of amassed cocci and fibriform extracellular matrix as seen Fig. 4a, b and c. The size, shape and mat formation of the biofilm forming bacteria were clearly seen. The cells were found to be attached on to the surface of coverslip which was immersed in the nutrient broth for 72 h and it confirms the production of extracellular polymeric substances. The formation of polymeric fibrils and enveloping matrix confirms that the extracellular polymeric substances lead to the biofilm formation (Eighmyi et al., 1983). Scanning electron microscopic images also reveal the bacterial cell shape and physical morphology of the biofilm forming bacteria.

Characterization of extracellular polymeric substance

The extracellular polymeric substances (EPS) produced by

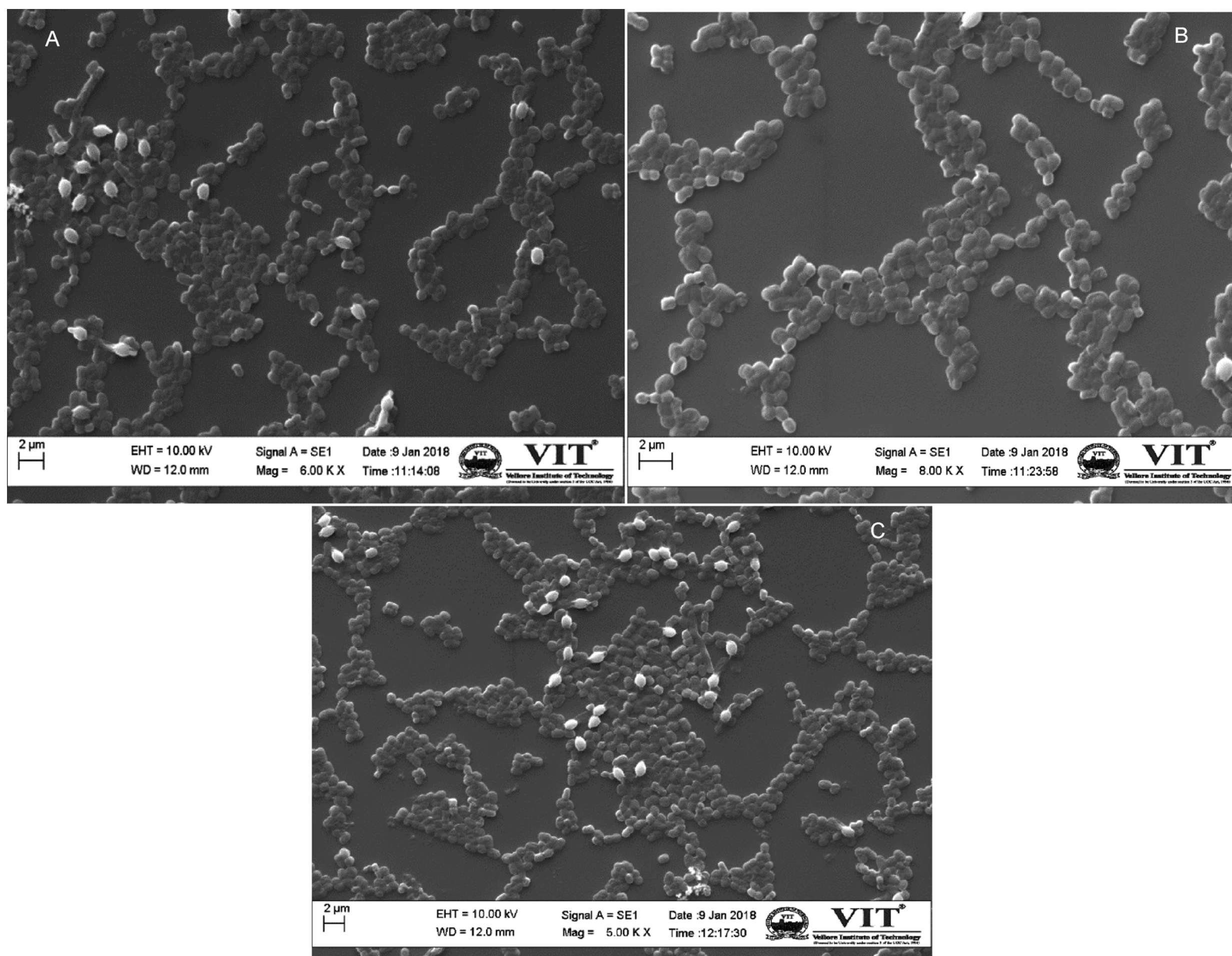

Figure 4 Scanning electron microscopic images (A) *P. celer*, (B) *P. alimentarius* and (C) *K. rhizophila*.

Table 2 Chemical analysis of extracellular polymeric matrix produced by biofilm forming bacteria

Bacterial isolates	Protein content (μg/ml)	Total carbohydrate content (μg/ml)
Isolate 3 (*P. celer*)	1152	102.5
Isolate 4 (*P. alimentarius*)	980	87.3
Isolate 8 (*K. rhizophila*)	1265	142.6

the biofilm forming bacteria were extracted and characterized. EPS is often a complex mixture of proteins, carbohydrates, lipids, DNA, and humic acid substances (Flemming et al., 2007) and hence its detailed composition analysis is challenging. In the present study the two major components of EPS viz. protein and total carbohydrate were estimated. The EPS fractions extracted from the biofilms formed by the three different isolates showed varied protein and carbohydrate content (Table 2). EPS from isolate 8 (*K. rhizophila*) had highest protein and carbohydrate content (1265 μg/ml and 142.6 μg/ml respectively), followed by isolate 3 which contained 1152 μg/ml of protein and 102.5 μg/ml of total carbohydrate respectively. Isolate 4 which had a comparatively less biofilm forming ability among the three isolates showed a similar trend in the composition of EPS as well.

The nature of carbohydrates within EPS varies with the microbial community within the biofilms. This is reflected by the complexity of carbohydrates containing diverse sugar residues extracted from biofilm matrices (Wrangstadh et al., 1990; Manca et al., 1996; Verhoef et al., 2002). Moreover, the role of polysaccharides in the development and function of biofilm matrices remains hypothetical.

Conclusion

Three bacterial isolates were isolated from marine biofilm samples collected from ship hull. The ability of these three isolates to produce biofilms was determined by microtiter plate assay. Further the isolates were characterized by biochemical tests and molecular characterization was carried out by 16S rRNA gene sequencing and the isolates were identified *as Psychrobacter celer*, *Psychrobacter alimentarius* and *Kocuria rhizophila*. The biofilm formation was characterized by Scanning electron microscopy and the EPS produced by the bacteria was extracted and the protein and carbohydrate content estimated.

Conflicts of interest

The authors declare that they have no conflict of interest.

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