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Extracellular polymeric substances from two biofilm forming *Vibrio* species: Characterization and applications

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ABSTRACT

The early biofilm colonizing *Vibrio campbellii* and *Vibrio fortis* produced 400 and 134 mg L^{-1} extracellular polymeric substances (EPS), respectively of distinct composition in planktonic cultures. The EPS of *V. campbellii* consisted of five monosaccharides: arabinose, galactose, glucose, mannose and rhamnose, however, that of *V. fortis* has only three monosaccharides: arabinose, galactose and mannose. The AFM topography analysis showed different morphology and physical characteristics of EPSs. FTIR and NMR spectral analyses revealed characteristic functional groups of the polysaccharides. EPSs were amorphous in nature and contained characteristic diffraction peaks. EPSs showed high emulsifying activity and pseudoplastic rheology. This is the first report of detailed characterization of EPS produced by *V. campbellii* and *V. fortis*, so far. Detailed analytical (FT-IR, ^{1}H NMR, PXRD, etc.) and bio-physicochemical characteristics (thermostability, emulsifying and rheological properties) of EPSs showed potential for industrial applications.

1. Introduction

The biofilm mode of life is a feature common to most microorganisms in natural, medical and engineered systems (McDougald, Rice, Barraud, Steinberg, & Kjelleberg, 2012). Biofilms are multicellular communities of microorganism, residing in hydrated extracellular polymeric substances (EPS) matrix produced by them. Biofilm is formed by interaction among early and late colonizers, however little is known about early stages of biofilm formation (Siboni, Lidor, Kramarsky-Winter, & Kushmaro, 2007). The EPS matrix contains unique constituents and is responsible for making biofilms, the most successful form of life on earth. Mostly in biofilm, microorganisms occupy only 10% of the dry mass, while rest 90% are occupied by matrix (EPS) (Flemming & Wingender, 2010). The EPS mainly consists of polysaccharides, proteins, extracellular DNA and lipids, which together make architecture for 'houses of biofilm cells' (Flemming, Neu, & Wozniak, 2007). This matrix helps organism to acclimatize with habitat and impart protection against stress such as desiccation, biocides, antibiotics, heavy metals and UV radiation etc. (Flemming & Wingender, 2010).

The EPS of biofilm forming microorganisms possess a wide diversity of structural, physical, rheological and other unique properties, which makes it renewable sources of biotechnological importance (Kavita, Mishra, & Jha, 2011; Vu, Chen, Crawford, & Ivanova, 2009). Structural diversity makes marine EPSs a potential source for commercial application including drug discovery, however this area of research is unexplored (Laurienzo, 2010). *Vibrio* species are well known for biofilm formation and are ubiquitous in marine ecosystems (Yildiz & Visick, 2009). Exopolymers produced by *Vibrio spp.* display huge diversity in composition and potential applications. Despite the immense potential of exopolymers produced by *Vibrio spp.*, the characterization of EPS by only few species, viz., *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio furnissii* and *Vibrio parahaemolyticus* was attempted (Bramhachari & Dubey, 2006; Bramhachari et al., 2007; Kavita et al., 2011; Muralidharan & Jayachandran, 2003). Moreover, the EPS production and their biotechnological properties vary not only at species level but also at the strain level.

In this study, the extracellular polymeric substances of two early colonizer (sharing the same niche) of marine biofilm were isolated, characterized, compared and their rheological, emulsifying properties were studied for its potential applications. Moreover, the biofilm formation ability and hydrophobicity test were done to have better insight of role of EPS in biofilm formation during early stage. Apart from detail characterization of EPSs of two biofilm forming species of *Vibrio* other bio-physicochemical properties were also studied to explore its industrial applications.

2. Materials and methods

2.1. Biofilm formation assay and growth curve

Overnight cultures of bacterial isolates were diluted to absorbance ($OD_{620\,nm}$) 0.1, loaded to microtiter plate (100 μL/well)

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and incubated for 24 h (180 rpm, 30 °C). Bacterial growth was measured ($OD_{620\,nm}$) and cultures were aspirated out carefully. Microtiter plate wells were washed (H_2O), dried and stained with 1% Crystal violet. Excess dye was washed off, ethanol (96% v/v, 200 μL) was added and the absorbance was measured at 590 nm (Andersson, Dalhammar, Land, & Rajarao, 2009). All assays were done in eight replicates.

2.2. Isolation and characterization of biofilm forming marine bacteria

Sterile plastic petri-dishes were submerged in Arabian Sea at Diu, India (latitude N 20°42′20.8″ and longitude E 70°58′6.42″) and early colonizer (bacteria) of biofilm were isolated after 12 h, using Zobell 2216 agar medium (Kwon et al., 2002; Kavita et al., 2011; Sweet, Croquer, & Bythell, 2011). Two bacterial strains (BK3 and BK4) were selected by biofilm formation assay and quantifying the amount of total EPS produced, for further studies. Bacterial strains were identified by 16S rRNA gene sequences, amplified using universal primers (fD1-5′-AGA GTT TGA TCC TGG CTC AG-3′ and rP2-5′-ACG GCT ACC TTG TTA CGA CTT-3′) and optimized PCR conditions (Weisburg, Barns, Pelletier, & Lane, 1991).

2.3. Extraction, purification and molecular weight determination of EPSs

Bacterial isolates were cultured in Zobell 2216 medium (500 mL) under controlled laboratory condition at 30 ± 2 °C (180 rpm) and EPSs were extracted using procedures, optimized previously (Kavita et al., 2011). EPS was dialysed for 1 day against distilled water for purification and purified EPS was lyophilized at −70 °C for 10–12 h (Kavita et al., 2011). The molecular weight of extracted EPS was determined by gel permeation chromatography (GPC; 7.8 mm ID × 300 mm stainless steel, Water Allaince, model 2695, Waters, USA). About 50 μL (2% w/v) purified EPS was loaded to GPC column (Ultrahydrogel −120 and 500) at 40 °C and elution was monitored by a refractive index detector (2414). The column was calibrated with standard dextran (molecular weight; 5200–668,000 kDa; PSS, USA) (Singh et al., 2011).

2.4. Energy dispersive X-ray spectroscopy (EDX) and emulsifying activity

Elemental analysis of EPS was done using energy dispersive X-ray spectroscopy (EDS or EDX; Oxford Instruments, UK) which revealed the weight and atomic percentage of different elements present (C, O, Na, S, and Ca) in the sample (Mishra, Kavita, & Jha, 2011). Emulsifying activity of EPS samples were measured with hexadecane and expressed as the percentage retention (t%) of emulsion during incubation for time 't' (Mishra & Jha, 2009).

2.5. Fourier-transformed infrared spectroscopy and nuclear magnetic resonance (NMR)

The major structural groups of purified EPS were detected using Fourier-transformed infrared (FT-IR) spectroscopy and ^{1}H NMR. The FT-IR spectra were recorded in the region of 4000–400 cm^{-1} on GX FT-IR system (Perkin-Elmer, USA) and KBr pellet was used as a background reference (Mishra & Jha, 2009). The ^{1}H NMR spectrum of EPS (5 mg/mL) was obtained in D_2O at 25 °C with Bruker Avance II 500 (Switzerland) spectrometer, operating at 500 MHz with net spinning 5000 rpm, 5.9 μs pulse duration, 1.2 s acquisition time and 6 μs relaxation delay (Jain, Mody, Mishra, & Jha, 2012a, 2012b).

2.6. Powder X-ray diffraction analysis

X-ray diffraction was performed on X-ray powder diffractometer (Philips X'pert MPD, The Netherlands) with 2θ ranging 2–80° at 25 °C. The irradiated length and specimen length were 10 mm with receiving slit size of 0.2 mm at a 200 mm goniometer radius. Distance between the focus and divergence slit was 100 mm. Dried EPS sample was mounted on a quartz substrate and intensity peaks of diffracted X-rays were continuously recorded with scan step time 1 s. The d-spacing of EPSs at that value of θ were calculated (Kavita et al., 2011; Mishra et al., 2011).

2.7. Analytical gas chromatography mass spectrometry (GCMS)

Purified EPSs were assayed for total carbohydrate content using the phenol sulfuric acid assay with glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Monosaccharide contents of EPS were estimated by alditol-acetate method. For this, the purified EPS was hydrolyzed with 2 M H_2SO_4 at 121 °C for 12 h, thereafter neutralized by adding $BaCO_3$ and filtered. Recovered concentrated sample-solution was subjected to reduction using $NaBH_4$ for 12 h and reduced sugar-solution was passed through activated resin for Na^+ removal. The sample-solution was filtered, methanol was added and kept to evaporate. The process was repeated three times and last 2 mL sample-solution (in round bottom flask) was kept in desiccator for crystal formation. For acetylation, pyridine and acetic anhydride were added in the flask containing crystals and reflux reaction was carried out at 100 °C for 30 min. The acetylation reaction was stopped by adding ultra pure water. Aqueous solution of ethyl acetate was added, mixed, separated (in separating funnel) and ethyl acetate part, containing aldol-acetate was collected. The collected sample-solution was neutralized by using saturated solution of Na_2CO_3 and pyridine was removed using saturated solution of $CuSO_4$. Aqueous impurities were removed by adding anhydrous Na_2SO_4 and recovered volume (containing aldol-acetate) was concentrated. Concentrated volume was dried to recover aldol-acetate in crystalline form. The monosaccharide composition of derivatised samples was analyzed and quantified on GCMS-QP2010 (Shimadzu, Japan) using SGE BP-225 capillary column (Siddhanta et al., 2001).

2.8. Atomic force microscopy (AFM) and scanning electron microscope (SEM)

The surface topography of EPS was observed under atomic force microscopy (AFM; Ntegra-Aura, NT-MDT, Moscow, Russia) in the tapping mode. The topographic AFM maps of EPSs were used to calculate roughness root mean square (R_q), surface skewness (R_{sk}), coefficient of kurtosis (R_{ku}), area root mean square slope (A_q) and functional indices like surface bearing index (S_{bi}), core fluid retention index (S_{ci}) and valley fluid retention index (S_{vi}). The morphology of bacteria, in both planktonic and sessile (attached with biofilm) forms, and EPSs (extracted from planktonic cultures) were observed under a scanning electron microscope (SEM, LEO series VP1430, Germany) with an accelerated voltage of 20 kV (Andersson et al., 2009; Kavita et al., 2011).

2.9. Thermal gravimetric (TG) and differential scanning calorimetry (DSC) analysis

TG and DSC scanning were carried in the range of 30–400 °C and 25–600 °C, respectively under nitrogen atmosphere at rise of 10 °C min^{-1}, using Mettler Toledo TGA/SDTA System (Greifensee, Switzerland). The Sample was enclosed in an aluminum vessel and its energy level was scanned. TG and DSC analysis were carried out by gradually raising the temperature, plotting weight

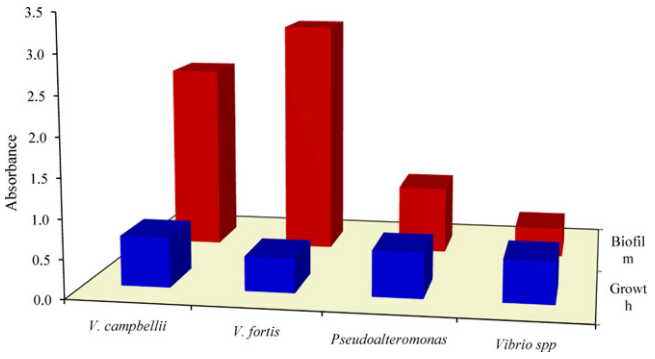

Fig. 1. Growth rate and biofilm formation of selected bacterial isolates.

(percentage) and heat flow against temperature, respectively (Mishra et al., 2011).

2.10. Rheological studies

Lyophilized samples were dissolved in distilled water (0.4% w/v) and dynamic rheological measurement was carried out on rheometer (Anton paar physica MCR 301, USA) using parallel plate PP50/P-PTD200 geometry (50 mm diameter; 0.1 mm gap) at different temperature, applied shear rate and pH with 1 mL sample volume (Mishra et al., 2011). Measurements were carried out immediately after placing the samples on plate and outer surface of the samples were covered with silicon oil to avoid loss due to evaporation at higher temperature. The viscosity was measured at 25 °C with different shear rates (50–950 s^{-1}). The influence of pH on the viscosity was studied by comparing viscosity at pH 3.0 and 7.0. The influence of temperature (10–60 °C) was analyzed at both pH. All experiments were carried out in triplicates and slippage of gel due to applied stress was carefully avoided by selecting appropriate operation parameters.

2.11. Microbial adhesion to hydrocarbons (MATH) assay

The MATH assay was carried out with 24 h grown cultures. Bacterial isolates were harvested, washed twice and resuspended in 150 mM KCl (pH 7) solution. Bacterial cells were adjusted to approximately 1.0 $OD_{595\,nm}$ with 150 mM KCl. About 1 mL cell suspension was taken in a sterile polystyrene tube and 200 μL of *n*-hexadecane was added. The mixture was incubated at 30 °C for 15 min, vortexed for 2 min and allowed to settle for 15 min at room temperature. The aqueous phase was taken carefully and absorbance was measured at 595 nm (Rosenberg, 1984).

3. Results and discussion

3.1. Biofilm formation assay

Total of ten isolates were obtained from biofilm and in order to select prominent early colonizer, biofilm formation ability of all isolates were analyzed. Four isolates (three *Vibrio* sp. and one *Pseudoalteromonas* sp.) were observed that they were involved in biofilm formation significantly (Fig. 1). Two *Vibrio* species (*V. campbellii* and *Vibrio fortis*) showed high biofilm formation and therefore selected for further study. These colonizers were considered early colonizer as they are associated with early stage (2–12 h) of biofilm (Sweet et al., 2011).

Table 1
Elemental EDX microanalysis of EPSs obtained from *Vibrio spp.*

Elements	EPS [*V. campbellii*]		EPS [*V. fortis*]	
	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
C	24.55	33.26	39.05	49.16
O	53.99	54.90	45.35	42.86
Na	2.58	1.82	1.83	1.20
Mg	8.18	5.47	4.33	2.70
Al	0.17	0.10	0.20	0.11
Si	0.06	0.04	0.35	0.19
P	1.00	0.52	3.46	1.69
S	4.19	2.13	2.65	1.25
Ca	3.60	1.46	1.26	0.47
Fe	0.07	0.02	0.60	0.16
Sr	0.95	0.18	0.74	0.13
In	0.66	0.09	–	–
K	–	–	0.18	0.07

Data are expressed as both weight and atomic percents.

3.2. Molecular identification of bacteria and quantitative determination of EPS

Bacterial isolates selected for further studies were identified by 16S rRNA gene sequence homology. Sequences (16S rRNA) of both isolates were subjected to NCBI blast. One isolate was identified as *Vibrio campbellii* showing 98% sequence homology (with 99% query coverage) with database. Another isolate, showing 99% sequence homology with 100% query coverage, was identified as *V. fortis*. The 16S rRNA gene sequences of both species were deposited in NCBI Genbank with the accession number JQ627648 and JQ627649, respectively.

Bacterial isolates were cultured in Zobell 2216 medium under controlled laboratory condition and high EPS production was observed in the late log phase. The growth of *V. campbellii* is higher than *V. fortis* and it was observed that high EPS was produced in *V. campbellii*, within 12 h (Fig. S1). About 400 and 134 mg L^{-1} EPS was extracted from planktonic culture of *V. campbellii* and *V. fortis*, respectively. The yield of EPS was significantly greater compared with that produced by other *Vibrio* species. Generally 30–60 mg L^{-1} EPS were extracted from *Vibrio* species; *V. harveyi*, *V. furnissii* and *V. parahaemolyticus* (Bramhachari & Dubey, 2006; Bramhachari et al., 2007; Kavita et al., 2011).

3.3. Energy dispersive X-ray spectroscopy (EDX) and emulsifying activity

Elemental quantitative analyses of EPSs were done by EDX which revealed the presence of weight and atomic percentage of different elements (Table 1 and Fig. S2). Sulphate residue was observed in exopolysaccharides produced by *V. campbellii* (4.19% w/w) compared to *V. fortis* (2.65% w/w). The emulsifying activities of exopolymers, obtained from both species of *Vibrio*, were determined by its strength in retaining the emulsion and it was found 64.28% up to 30 min thereafter decreased to 52.43% at 60 min for *V. campbellii*. EPS extracted from *V. fortis* showed emulsifying activity 71.64% up to 30 min and intend to decline 51.54% for next 30 min. The emulsion of exopolymers were found unstable compared to *V. parahaemolyticus* (67.37% for 60 min), while stable compared to that of other EPS (34–40%), produced by *V. harveyi* VB23 (Bramhachari & Dubey, 2006; Kavita et al., 2011).

3.4. Fourier-transformed infrared spectroscopy and nuclear magnetic resonance (NMR)

The FTIR spectra of EPSs, obtained from *Vibrio* cultures, revealed characteristic functional groups (Fig. 2). The broad stretching peak of hydroxyl group was detected (frequency

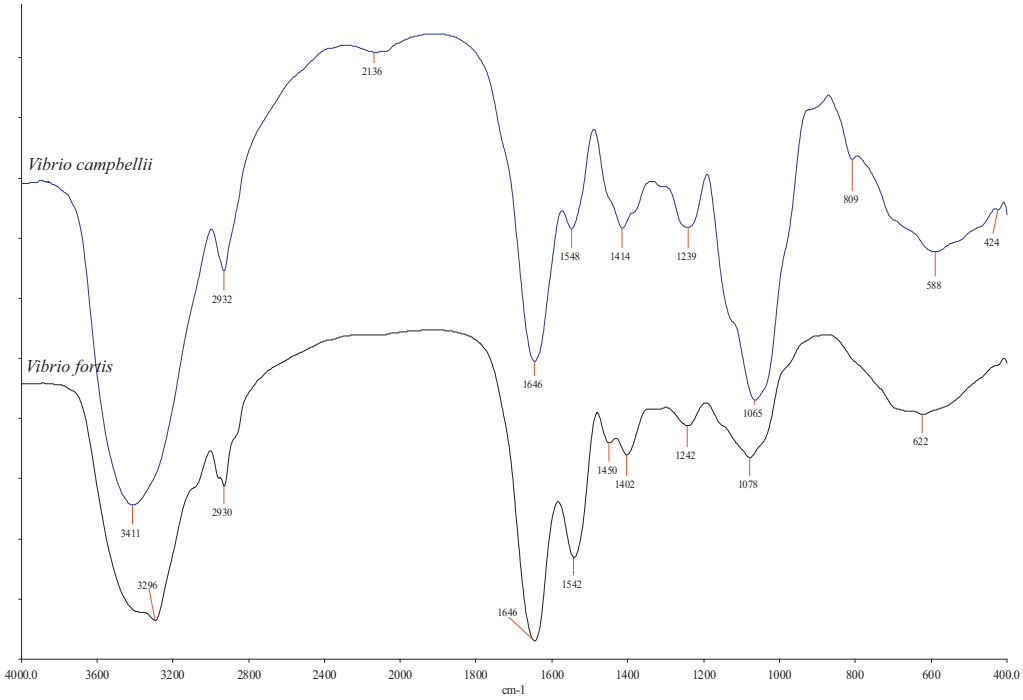

Fig. 2. FT-IR spectra of EPSs extracted from *Vibrio spp.*

range 3600–3200 cm^{-1}) in EPS (Nwodo & Okoh, 2012) extracted from both *Vibrio* spp. Weak absorption at 2930/2932 cm^{-1} (2915–2935 cm^{-1}), detected in both EPSs was attributed to asymmetrical C—H stretching vibration of aliphatic CH_2 group, which revealed presence of sugar (Iyer, Mody, & Jha, 2005) or protein content. Similarly, an asymmetrical medium stretching peak, corresponded to the ring stretching of galactose and mannose was observed at 1646 cm^{-1} (1593–1662 cm^{-1}) which corresponded to the ring stretching of galactose and mannose (Freitas et al., 2009). Amide II vibration in combination with C—N stretching mode was detected near IR peak 1548/1542, however peak near at 1414/1402 cm^{-1} represented the symmetric stretching of —COO^- group. Absorption peak in the range of 1000–1125 cm^{-1} confirmed the presence of uronic acid (O-acetyl ester linkage bonds) (Bramhachari & Dubey, 2006). The peak between 1200 and 900 cm^{-1} was attributed to polysachharide composition of EPS (Cao et al., 2011). Peaks around 690–515 cm^{-1} correspond to N—H wag of primary amine and C—X stretch of alkyl halides, respectively (Mishra & Jha, 2009). A peak of 1239/1242 was detected for the C—N stretch (1250–1020 cm^{-1}) of aliphatic amines. Absorption peaks in the range of 910–665 cm^{-1} correspond to N—H wag of primary amine and a weak peak at 2136 cm^{-1} were observed only in the EPS extracted from *V. campbellii*. The ^1H NMR spectra of EPSs extracted from *Vibrio spp.* exhibited characteristic chemical shifts (ppm) and corresponding functional groups (Fig. 3). A strong signal of water (HOD) was detected at ppm 4.8 and a signal corresponding to uronic acid is observed at 3.7 ppm (while, acetyl amine of hexose or pentose was detected at about 2.0 ppm, however peaks at 2.6 ppm confirmed the presence of H—C—COOH functional group of proteins (Jacobsen, 2007; Kavita et al., 2011; Mishra et al., 2011). NMR spectral analysis showed EPSs of both *Vibrio* species comprised of almost similar functional groups as also observed previously for *V. parahaemolyticus* (Kavita et al., 2011). The presence of acetyl groups evidenced that EPSs are hydrophobic in nature.

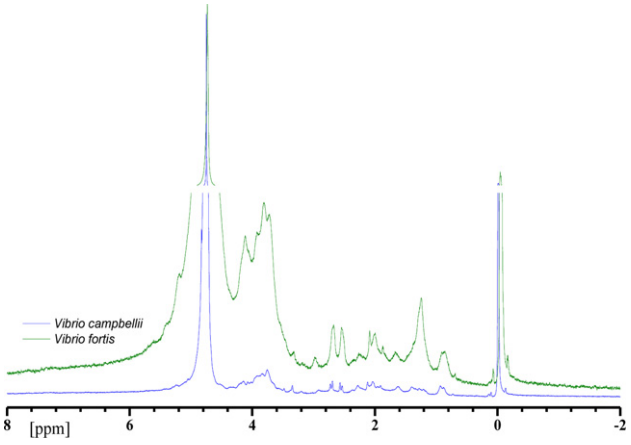

Fig. 3. Proton nuclear magnetic resonance (^1H NMR) spectra of EPSs obtained from *Vibrio spp.*

3.5. Powder X-ray diffraction analysis

A rapid analytical technique, X-ray powder diffraction (XRD) is used for the phase identification of EPSs obtained from *Vibrio* species (Fig. S3). Characteristic diffraction peaks at 5.617, 9.4391 and 28.6648, with interplanar spacings (d-spacings) at 15.07791, 9.36989 and 3.11430 Å, respectively were observed in XRD analysis of EPS extracted from *V. campbellii*. The XRD profile of the EPS obtained from *V. fortis* exhibited diffraction peaks at 5.9575, 9.4730 and 28.6598, with d-spacings at 14.83551, 9.33639 and 3.11484 Å, respectively. The PXRD profile was attributed characteristic pattern to the EPS obtained from *V. campbellii, V. fortis* and *V. parahaemolyticus* (Kavita et al., 2011).

3.6. Molecular mass and monosaccharide composition

Molecular mass of EPSs extracted from *V. campbellii* and *V. fortis* was found to be 2431 (with 1.19 polydispersity) and 2218 kDa (with 1.15 polydispersity) respectively with 16.1 min retention time (Fig. S4). The GPC chromatogram generated single peaks for the both EPSs. In general, bacterial EPSs consist of average

molecular mass 2000 kDa (Jain et al., 2012a, 2012b; Singh et al., 2011; Singh, Shukla, Mishra, Reddy, & Jha, 2013). However, high molecular masses are also reported for the bacterial EPSs (Mata et al., 2008; Singh et al., 2011, 2013). Total sugar content was found to be 183.6 and 210.14 μg/mg EPS in the EPS extracted from *V. campbellii* and *V. fortis*, respectively. The monosaccharide compositions of the EPS extracted from *V. campbellii* revealed the presence of five monosaccharides: arabinose (34.39 mol%), galactose (23.99 mol%), glucose (8.15 mol%), mannose (28.72 mol%) and rhamnose (4.73 mol%). EPS obtained from *V. fortis* comprised of three monosaccharide units; arabinose (51.37 mol%), galactose (30.73 mol%) and mannose (17.89 mol%). The mol percentage of arabinose was found maximum in both EPSs while glucose and rhamnose were detected only in EPS of *V. campbellii*. EPS of *V. campbellii* was found rich in monosaccharide compared to EPS of *V. fortis*. Cell surface interaction for biofilm development is mediated by EPS (Nielsen, Li, & Halverson, 2011; Yang et al., 2011) and thus sugar composition may play an important role in biofilm efficiency. Previously, four monosaccharides; arabinose, galactose, glucose and high mannose content were detected in EPS of *V. parahaemolyticus* (Kavita et al., 2011) while EPS produced by *V. harveyi* strainVB23 contained galactose and glucose as major sugars (Bramhachari & Dubey, 2006). Three monosaccharides galactose, glucose and mannose were constituted EPS, produced by *V. cholerae* (Wai, Mizunoe, Takade, Kawabata, & Yoshida, 1998). Different growth habitat determines the quality and quantity of polysaccharide formation (Cerning et al., 1994). GCMS analysis exhibited heteropolysaccharide nature of EPSs extracted from *Vibrio spp.*

3.7. Thermal gravimetric (TG) and differential scanning calorimetric (DSC) analysis

The thermal behavior of an exopolymer plays a key role for its commercial applicability. Degradation of EPSs extracted from *V. campbellii* and *V. fortis* takes place in two steps, about 57% and 37% EPS are degraded up to 270 °C and 280 °C, respectively. Second phase of depolymerisation (17% and 26%) occurred up to 350 °C (Fig. 4 a). Exopolymer obtained from *V. fortis* with 37% weight loss was observed thermo-stable compared to EPS of *V. campbellii* showing 57% degradation. In the previous study, EPS extracted from *V. parahaemolyticus* showed 13% weight loss at 160 °C (Kavita et al., 2011), hence cannot be considered thermo-stable compared to EPS obtained from these two *Vibrio* species. An exothermic process of transition from amorphous to crystalline solid was studied using differential scanning calorimetric analysis (Fig. 4b). DSC thermogram showed broad peak range of crystallization temperature (T_c) (80–125 °C) for EPS extracted from *V. campbellii* while EPS of *V. fortis* showed 99.08 °C crystallization temperature. Crystallization was followed by transition of melting and EPS of *V. campbellii* showed two peak of melting; first melting temperature (T_{m1}) was found at 360.297 °C and second transition (melting, T_{m2}) was detected at 475.91 °C, while *V. fortis* showed single melting transition peak at 338.97 °C. DSC thermogram of the EPSs extracted from both *Vibrio spp*. showed high thermo-stability and almost similar thermogram was observed with EPS extracted from *V. parahaemolyticus* (Kavita et al., 2011). Thermo-stability of the extracted EPSs makes it a promising additive for the industrial applications.

3.8. Scanning electron microscope (SEM) and atomic force microscopy (AFM)

SEM analysis showed that biofilm produced by *V. campbellii* is smooth, compact and firmly attached with bacteria, while biofilm of *V. fortis* is loosely bound with bacteria and irregular in shape (Fig. S5). It has been clear from SEM images of EPSs that exopolymer extracted from *V. campbellii* asymmetric compared to EPS obtained

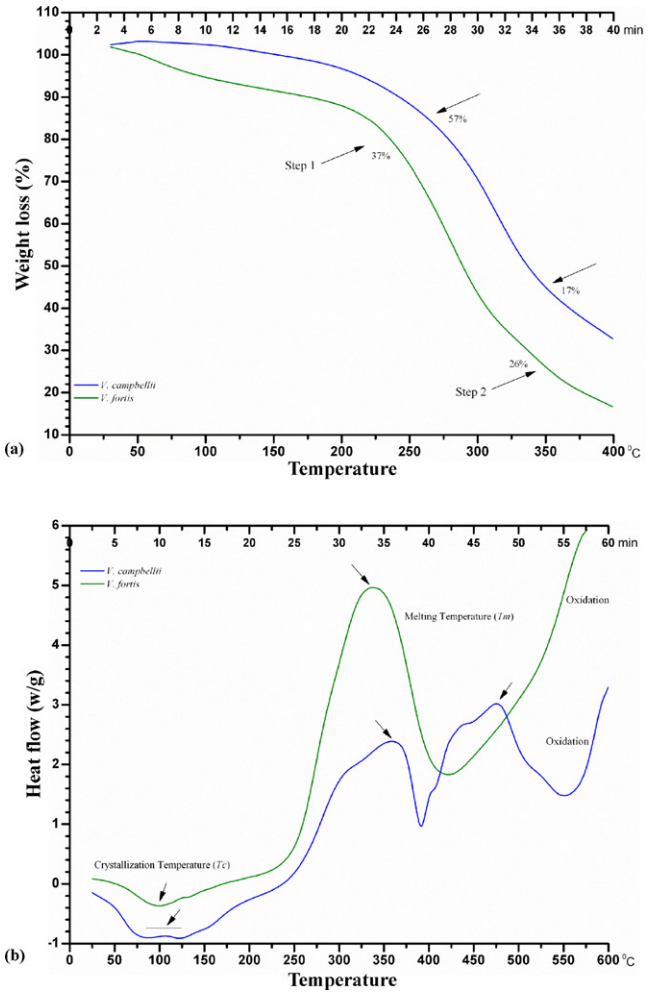

Fig. 4. Thermogravimetric (a) and DSC (b) thermogram of EPSs extracted from *Vibrio spp*.

from *V. fortis* (Fig. S6). The exopolymer of *V. fortis* was compact in nature with small pores. Compact EPS with small pore size distribution was also obtained from *V. parahaemolyticus* (Kavita et al., 2011). Physical characteristics of exopolymer were determined by measuring surface morphology using AFM (Fig. S7). From AFM topographic maps of EPSs, different morphological parameters and functional indices were calculated (Table 2). AFM topography analysis exhibited different morphology and functional characteristic of EPSs, produced by early colonizers *Vibrio spp*., sharing common habitat and environmental conditions during biofilm formation.

3.9. Rheological studies

The dynamic viscosity (η) of EPSs decreased concomitantly with shear rate (γ) up to 97.3 shear rate (s^{-1}) and showing pseudoplastic rheological property at constant temperature, 25 °C and

Table 2
Surface morphology parameters and functional indices of EPSs.

Characteristics	EPS [*V. campbellii*]	EPS [*V. fortis*]
Roughness root mean square (R_q)	0.136 μm	0.196 μm
Surface skewness (R_{sk})	0.676	0.372
Coefficient of kurtosis (R_{ku})	6.270	3.533
Area root mean square slope (A_q)	0.202	0.427
Surface bearing index (S_{bi}),	0.24	0.85
Core fluid retention index (S_{ci})	1.59	1.41
Valley fluid retention index (S_{vi})	0.13	0.14

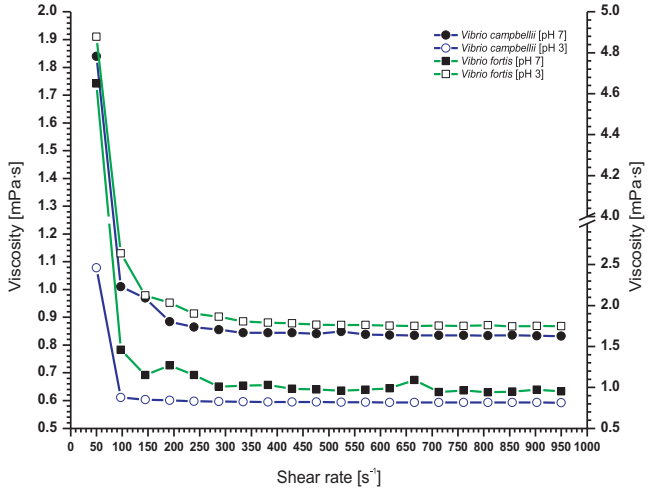

Fig. 5. Rheology of EPSs: effect of shear rate on viscosity of EPSs isolated from *Vibrio spp.* at constant temperature (25 °C).

different pH (Fig. 5). Viscosity also decreased concurrently with temperature at both pH and constant shear rate, 50 s^{-1} (Fig. S8). Interestingly, similar rheological behavior of EPSs, obtained from *V. campbellii* and *V. fortis* was observed at different pH. EPS of *V. campbellii* showed same rheology as the EPS of *V. fortis* at pH 3 and 7, respectively. Similarly, EPS of *V. fortis* exhibited alike rheology as the EPS of *V. campbellii* at pH 3 and 7, respectively. EPS of *V. fortis* showed more viscosity compared to *V. campbellii* at constant temperature, whereas reverse i.e. high viscosity for *V. campbellii* compared to *V. fortis* was observed at constant shear rate for pH 3 and 7. Previously, it was observed that viscosity of EPSs obtained from *V. parahaemolyticus* and *V. alginolyticus* decreased concomitantly with shear rate and temperature, respectively (Kavita et al., 2011; Muralidharan & Jayachandran, 2003). The viscosity of the EPS extracted from *V. alginolyticus* was unstable at high temperatures and high pH, decreased throughout a range of increasing shear rate and temperature (Muralidharan & Jayachandran, 2003). Rheology independent from pH was observed for EPS extracted from *V. parahaemolyticus* (Kavita et al., 2011). EPSs extracted from halophilic species exhibited low viscosity and pseudoplastic behavior (Mata et al., 2008).

3.10. Measurement of cell surface hydrophobicity

The hydrophobicity of the cell surfaces is an important factor for the attachment of cells to a surface. The surface hydrophobicity of cell was determined by the MATH assay (Rosenberg, 1984) and results reveal the moderate hydrophobic nature of *V. campbellii* (65.1%) and low hydrophobicity (43.8%) of *V. fortis* cell surface. MATH assay, formerly known as BATH assay is commonly used for the measurement of bacterial cell surfaces (Karunakaran & Biggs, 2011; Rosenberg, 2006; Thwaite, Laws, Atkins, & Atkins, 2009) and the removal of more than 50% of the cell suspension into the organic phase from the aqueous phase is considered for hydrophobic nature of cell surface (Karunakaran & Biggs, 2011).

4. Conclusion

In the present study, two early colonizer *Vibrio* species (*V. campbellii* and *V. fortis*) were isolated from artificial marine biofilm. *V. campbellii* is fast growing, produce more EPS but less efficient in biofilm formation as compared to *V. fortis*. The study was first report on detailed analytical and bio-physicochemical study of EPS produced by *V. campbellii* and *V. fortis* so far. The analysis showed different composition and morphology of EPSs. The study evident that different species of *Vibrio* in a biofilm have different EPS constituents and varied biofilm formation capability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.02.010.

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