Isolation and physico-chemical characterisation of extracellular polymeric substances produced by the marine bacterium *Vibrio parahaemolyticus*

Kumari Kavita, Avinash Mishra and Bhavanath Jha*

Discipline of Marine Biotechnology and Ecology, Central Salt and Marine Chemicals Research Institute, Council of Scientific and Industrial Research (CSIR), G. B. Marg, Bhavnagar – 364021, Gujarat, India

(Received 28 August 2010; final version received 7 February 2011)

A marine bacterial strain identified as *Vibrio parahaemolyticus* by 16S rRNA gene (HM355955) sequencing and gas chromatography (GC) coupled with MIDI was selected from a natural biofilm by its capability to produce extracellular polymeric substances (EPS). The EPS had an average molecule size of 15.278 µm and exhibited characteristic diffraction peaks at 5.985, 9.150 and 22.823, with d-spacings of 14.76661, 9.29989 and 3.89650 Å, respectively. The Fourier-transform infrared spectroscopy (FTIR) spectrum revealed aliphatic methyl, primary amine, halide groups, uronic acid and saccharides. Gas chromatography mass spectrometry (GCMS) confirmed the presence of arabinose, galactose, glucose and mannose. 1HNMR (nuclear magnetic resonance) revealed functional groups characteristic of polysaccharides. The EPS were amorphous in nature (CI$_x$rd 0.092), with a 67.37% emulsifying activity, thermostable up to 250°C and displayed pseudoplastic rheology. MALDI-TOF–TOF analysis revealed a series of masses, exhibiting low-mass peaks (m/z) corresponding to oligosaccharides and higher-mass peaks for polysaccharides consisting of different ratios of pentose and hexose moieties. This is the first report of a detailed characterisation of the EPS produced by *V. parahaemolyticus*, which could be further explored for biotechnological and industrial use.

Keywords: DSC; EPS; MALDI-TOF-TOF; *Vibrio*; XRD

Introduction

All surfaces exposed to the marine environment are subject to colonisation by marine organisms, a phenomenon known as biofouling, comprising microfouling and macrofouling (Aldred and Clare 2008; Dobretsov et al. 2009). In an aqueous environment, microbial cells grow in association with surfaces, leading to the formation of biofilms. Bacterial biofilms are sessile communities of microorganisms that coexist with highly differentiated extracellular polymeric substances (Dickschat 2010). The extracellular polymeric substances (EPS) are metabolic products that accumulate on the microbial cell surface and provide protection to the cells by stabilising their membrane structures against the harsh external environment and also serve as carbon and energy reserves during starvation. EPS, a heterogeneous matrix of polymers composed of polysaccharides, proteins, nucleic acids and (phospho)lipids (McSwain et al. 2005; Flemming et al. 2007), are renewable resources of biotechnological importance. The precise composition of a biofilm varies with the resident species and environmental conditions.

The EPS of microorganisms possess a wide diversity of structural, physical, rheological and other unique properties (Vu et al. 2009). These exopolymers are used in food industries as thickeners and gelling agents to improve food quality and texture. In the pharmaceutical industry, exopolymers can be used as a hydrophilic matrix for the controlled release of drugs, the development of bacterial vaccines and to enhance nonspecific immunity (de Carvalho and Fernandes 2010). EPS are regarded as an abundant source of structurally diverse polysaccharides, some of which may possess unique properties for special applications such as sludge settling and dewatering (Subramanian et al. 2010).

Microbial EPS are preferred in industry owing to their novel functionalities, reproducible physicochemical properties, stable cost and abundant supply. The recent increase in demand for natural polymers in various industrial applications has motivated interest in developing new sources of EPS production. Marine microorganisms, which are currently used as a source of products of high aggregate value, such as pigments, metabolites, fatty acids and proteins, could also be
exploited for their EPS as biosurfactants and/or bioemulsifiers (Satpute et al. 2010). Marine microorganisms such as Acinetobacter, Arthrobacter, Pseudomonas, Halomonas, Myroides, Corynebacterium, Bacillus, Alteromonas spp. have been studied for the production of biosurfactants and exopolysaccharides (Satpute et al. 2010).

Most of the marine microbial world remains unexplored due to the enormity of the marine biosphere (Satpute et al. 2010). The discovery of potent biofilm-producing marine microorganisms could enhance the use of environmentally biodegradable EPS molecules in industry and reduce dependence on biohazardous, nondegradable synthetic polymers. To date, the study of biofilm-forming bacterial species has been scanty, especially from the Indian west coast, and exopolymer-producing marine bacteria have rarely been cultured or identified. Vibrio species are ubiquitous in marine ecosystems and well known for biofilm formation (Yildiz and Visick 2008). Exopolymers produced by Vibrio spp. display huge diversity in composition and potential applications. Despite the immense potential of exopolymers produced by Vibrio spp., the characterisation of EPS by only a few species, viz., Vibrio harveyi, Vibrio alginolyticus and Vibrio furnissii (Muralidharan and Jayachandran 2003; Bramhachari and Dubey 2006; Bramhachari et al. 2007), has been reported so far. The present study is the first report of a detailed characterisation of planktonic EPS produced by the marine bacterium Vibrio parahaemolyticus. This work involved the isolation and physiochemical characterisation of planktonic EPS extracted from the biofilm-forming V. parahaemolyticus, which could be useful in biotechnological and industrial applications.

Materials and methods

Isolation and identification of a biofilm-forming bacterial strain

Bacteria were isolated from a natural biofilm collected from the coastal region of Diu, India (latitude N 20° 42′ 20.8″, longitude E 70° 58′ 6.42″) using Zobell 2216 agar medium (Kwon et al. 2002). The maximum EPS-producing bacterial strain was selected by quantifying the amounts of total EPS produced, as identified by fatty acid methyl ester profiling using MIDI (GC system-6850, Agilent technologies, USA) and 16S rRNA gene sequencing amplified by universal primers (fD1–5′-AGA GTT TGA TCC TGG CTC AG -3′ and rP2–5′-ACG GCT ACC TGG TTA CGA CTT -3′) (Weisburg et al. 1991). The sequences were evaluated and phylogenetic relationships were inferred by a neighbour joining (NJ) method in BLAST (Zhang et al. 2000).

Extraction and purification of EPS

V. parahaemolyticus was cultured in Zobell 2216 medium (500 ml) under controlled laboratory conditions at 30 ± 2°C (180 rpm). Bacterial cultures (3 days old) were centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was filtered twice and concentrated to a volume of 100 ml using a rotary evaporator (Buchi, Switzerland) for 8–10 h. The exopolymer was precipitated with trichloroacetic acid (20%, 4°C, 30 min) to remove proteins and nucleic acids (Andersson et al. 2009). The supernatant was gradually precipitated by adding two volumes of cold isopropanol and held at 4°C for 12 h to obtain exopolysaccharides from crude EPS (Mishra and Jha 2009). The precipitate was washed with absolute alcohol and dissolved in 50 ml MilliQ water (Millipore, USA). Dissolved EPS were dialysed for 1 day against distilled water for purification, and purified EPS were lyophilised at –70°C for 10–12 h (Chi et al. 2007).

Particle size measurement and emulsifying activity

EPS (50% w/v) samples dissolved in water were sonicated for 5 min, and the particle size distributions were measured by laser diffraction (Malvern Mastersizer 2000, Malvern Ltd, Worcestershire, UK). Lyophilised EPS (1 mg) was dissolved in 0.5 ml deionised water, heated to 100°C for 15 min and allowed to cool to room temperature (25 ± 2°C). The volume was brought up to 2 ml using phosphate-buffered saline (PBS). The sample was vortexed for 1 min after the addition of 1 ml hexadecane. The absorbance at 540 nm was measured immediately before and after vortexing (A0). The decline in absorbance was recorded after incubation at room temperature for 30 min (At). A control was run simultaneously with 2 ml PBS and 1 ml hexadecane. The emulsification activity was expressed as the percentage retention (t%e) of emulsion during incubation for time t, following Equation (1) (Mishra and Jha 2009).

\[ t%e = \left( \frac{A_t}{A_0} \right) \times 100. \]  

Fourier-transform infrared spectroscopy

The major structural groups of purified EPS were detected using FTIR spectroscopy. The pellet for infrared analysis was obtained by grinding a mixture of 2 mg EPS with 200 mg dry KBr followed by pressing the mixture into a 16 mm diameter mould. The FTIR spectra were recorded in the region of 4000–400 cm⁻¹ on a GX FTIR system (PerkinElmer, USA).
**Powder X-ray diffraction analysis**

EPS (2 mg) were analysed by X-ray diffraction using an X-ray powder diffractometer (Philips X’pert MPD, The Netherlands) with 2θ ranging from 2 to 80° at 25°C. The irradiated length and specimen length were each 10 mm, with a receiving slit size of 0.2 mm at a 200 mm goniometer radius. The distance between the focus and divergence slit was 100 mm. Dried EPS samples were mounted on quartz substrata, and the intensity peaks of diffracted X-rays were continuously recorded with a scan step time of 1 s; d-spacings appropriate to the diffracted X-rays at each θ value were calculated from Bragg’s law (Equation 2).

\[ d = \frac{\lambda}{2\sin\theta} \]  

where θ is half of the scattering angle measured from the incident beam.

The crystallinity index (\( CI_{xrd} \)) was calculated as the ratio of the peak areas of the crystalline phases to the sum of the areas of crystalline peaks and the amorphous profile (Equation (3), Ricou et al. 2005).

\[ CI_{xrd} = \frac{\sum A_{\text{crystal}}}{\sum A_{\text{crystal}} + \sum A_{\text{amorphus}}} \]  

**Analytical gas chromatography mass spectrometry (GCMS)**

Exopolymers were assayed for total carbohydrate content using the phenol sulphuric acid assay with glucose as a standard (Dubois et al. 1956). The EPS were hydrolysed in 2.0 M H₂SO₄ (100°C, 6–8 h), and sugar was converted to alditol acetates by reduction followed by methylation (Mehta et al. 2010). Alditol acetates were analysed and quantified on a GCMS-QP2010 (Shimadzu, Japan) using an SGE BP-225 capillary column (25 m length, 0.22 mm diameter and 0.25 μm thickness). The injector temperature was 220°C, and the carrier gas was helium at a flow rate of 1.0 ml min⁻¹; the initial temperature of 160°C was held for 3.0 min, followed by a ramp from 160 to 230°C at 10°C min⁻¹ and a final hold at 230°C for 10 min, all at a pressure of 131.8 kPa. The injection volume was 1 μl, the electron impact ionisation was 70 eV, the temperatures of the ion source and quadrupole were both 240°C, and the acquisition mode was scanning from m/z 60 to m/z 400 with a scan interval of 0.5 s and a scan speed of 714 per cycle.

**MALDI TOF–TOF mass spectrometry**

EPS samples were prepared in acetonitrile (5% v/v; 1 mg ml⁻¹) and mixed with an equal volume of the matrix (α-cyano-4-hydroxycinnamic acid; 10 mg ml⁻¹). Matrix-assisted laser desorption/ionisation–tandem time-of-flight (MALDI TOF–TOF) analysis was performed on an Applied Biosystems 4800 MALDI TOF–TOF analyser with an Nd–YAG (neodymium-doped yttrium aluminium garnet) laser (355 nm, 200 Hz) operated at an acceleration voltage of 20 kV. Each spectrum was collected in positive ion reflector as well as linear mode, with an average of 1400 laser shots per spectrum (Mishra et al. 2011). The reproducibility of the spectra was determined from six spot sets in each mode, and the spectra were analysed after centroid and deisotoping using the Data Explorer software (Applied Biosystems, USA).

**Nuclear magnetic resonance (NMR) and scanning electron microscopy (SEM)**

The 1HNMR spectra of the EPS were obtained in D₂O with a Bruker Avance II 500 (Switzerland) operating at 500 MHz with net spinning at 5000 rpm. The sample was dissolved in water and dehydrated by sequential transfer to increasing concentrations of acetone up to anhydrous, and the morphology of the exopolymer was observed under a scanning electron microscope (SEM, LEO series VP1430, Germany) with an acceleration voltage of 20 kV.

**Thermal gravimetric (TG) and differential scanning calorimetry (DSC) analysis**

TG and DSC analyses of EPS were carried out on a Mettler Toledo TGA/SDTA System (Greifensee, Switzerland). The EPS were enclosed in an aluminium vessel, and their energy level was scanned in the ranges of 30–400°C and 25–600°C for TGA and DSC, respectively, under a nitrogen atmosphere at a heating rate of 10°C min⁻¹. TG and DSC analyses were performed by gradually raising the temperature and plotting the weight (percentage) and heat flow, respectively, vs temperature. The activation energy (\( E_a \)) was determined from the Arrhenius equation (Equation (4)):

\[ E_a = RT\ln\left(\frac{k}{A}\right) \]  

where \( A \) is the frequency factor for the reaction, \( R \) is the universal gas constant, \( T \) is the temperature (in Kelvin) and \( k \) is the reaction rate coefficient.

**Rheological studies**

Lyophilised samples were dissolved in distilled water (0.4% w/v) (Khattar et al. 2010), and their viscosities
were measured on a rheometer (RS1, Haake Instruments, Karlsruhe, Germany) at 25°C at varying shear rates (50 to 950 s⁻¹). The influence of pH on the viscosity was studied by comparing the viscosities at pH 3.0 and 7.0 (Béjar et al. 1998). The influence of temperature (10°C to 60°C) was analysed at both pH values (Checa et al. 2007). All experiments were performed in triplicate, and slippage of the gel due to applied stress was carefully avoided by selecting appropriate operating parameters.

Results and discussion

Molecular identification of bacterial strain and EPS extraction

A total of 11 different types of bacterial colonies were obtained from the natural biofilm on Zobell agar medium, among which one isolate was selected on the basis of EPS production (Gauri et al. 2009). Using conserved primers (Weisburg et al. 1991), a 1.42 kb 16S rRNA gene was amplified, sequenced and submitted to NCBI (GenBank Accession no. HM355955). Phylogenetic analysis of the 16S rRNA gene sequence revealed a close resemblance with V. parahaemolyticus (Supplementary Figure S1 [Supplementary material is available via a multimedia link on the online article webpage]). Fatty acid methyl ester profiling of the screened bacterial strain further confirmed that the isolated bacterium was V. parahaemolyticus (Supplementary Figure S2 [Supplementary material is available via a multimedia link on the online article webpage]). High EPS production was observed in the late log phase and stationary growth phase; 58.98 mg l⁻¹ EPS was extracted from a V. parahaemolyticus culture grown in ZMB (Zobell marine broth) medium for 3 days. The yield of EPS was significantly greater compared with that produced by other Vibrio species; generally 27–30 mg l⁻¹ EPS can be extracted from Vibrio species (Bramhachari and Dubey 2006; Bramhachari et al. 2007).

Particle size and emulsifying activity

The EPS consisted of particles with sizes ranging from 4.394 (d0.1) to 40.248 (d0.9) μm, with an average size of 15.278 μm (d0.5) and a specific surface area of 0.728143 m² g⁻¹ (Supplementary Figure S3 [Supplementary material is available via a multimedia link on the online article webpage]). The emulsifying activity of the exopolymer was determined by its strength in maintaining an emulsion. It was found to be 67.37% stable for up to 60 min, a value that decreased to 60.52% at 90 min. The exopolymer emulsion was more stable than that of the EPS produced by V. harveyi VB23, which had a stability of 34–40% (Bramhachari and Dubey 2006).

FTIR spectroscopy of the EPS

Fourier-transform infrared spectroscopy (FTIR) reveals the specific frequencies at which molecules can rotate or vibrate, and these resonant frequencies are determined by the nature of molecules with associated vibronic coupling. The interpretation of infrared spectra involves the correlation of absorption bands in the spectrum of an unknown compound with the known absorption frequencies of different types of bonds. The FTIR spectrum of the EPS obtained from the V. parahaemolyticus culture revealed characteristic functional groups (Figure 1).

The broad stretch of frequency ranging from 3600–3200 cm⁻¹ was assigned to the hydroxyl group. The weak absorption at 2928 cm⁻¹ (2915–2935 cm⁻¹) was attributed to the asymmetrical C–H stretching vibration of an aliphatic CH₂ group, which revealed the presence of sugar content (Iyer et al. 2005). IR peaks observed in the range of 2350–2360 cm⁻¹ may be due to CO₂ adsorption or the asymmetric stretching of the –N=C=O– group (Mishra and Jha 2009). An asymmetrical medium stretching peak was observed at 1642 cm⁻¹ (1593–1662 cm⁻¹), which corresponds to the ring stretching of galactose and mannose (Freitas et al. 2009); however, a peak at 1416 cm⁻¹ represented the symmetric stretching of the –COO⁻ group (Pongjanyakul and Puttipipatkhachorn 2007; Freitas et al. 2009). The stretching of C–O–C and C–O at 1000–1200 cm⁻¹ corresponds to the presence of carbohydrates (Mishra and Jha 2009), and a peak at 1070 cm⁻¹ (1000–1125 cm⁻¹ range) confirmed the presence of uronic acid (O-acetyl ester linkage bonds) (Bramhachari and Dubey 2006). The absorption peaks at approximately 910–665 cm⁻¹ and 690–515 cm⁻¹
correspond to the N–H wag of primary amines and the C–X stretch of alkyl halides, respectively (Mishra and Jha 2009). The FTIR spectrum of EPS confirmed the presence of aliphatic methyl groups, primary amines, halide groups, uronic acid and saccharides (viz., galactose and mannose).

**Powder X-ray diffraction analysis**

X-ray powder diffraction (XRD) analysis is extensively used for the phase identification of polymers. The XRD profile of the EPS obtained from *V. parahaemolyticus* (Figure 2) exhibited characteristic diffraction peaks at 5.985°, 9.150° and 22.823°, with interplanar spacings (d-spacings) at 14.7666, 9.2998 and 3.8965 Å, respectively. The XRD pattern indicated that the EPS were amorphous in nature, with a CI of 0.092. The 9.2% content of crystalline domains acted as a reinforcing grid and improved the performance of the EPS over a wide temperature range, as observed in the calorimetric analysis. The XRD profile and interplanar spacing (d-spacing) are the basic characteristics of a polymer and are useful for comparing or studying the nature of EPS isolated from different sources.

**Monosaccharide composition by GCMS and MALDI TOF-TOF**

The content of total sugars was estimated by the procedure of Dubois et al. (1956) and was found to be 78.48 µg [mg EPS]⁻¹. The monosaccharide composition of the extracted EPS was analysed; GCMS analysis (Figure 3 and Supplementary Figure S4 [Supplementary material is available via a multimedia link on the online article webpage]) revealed the presence of four monosaccharides: arabinose (7.9 µg [mg EPS]⁻¹), galactose (19.2 µg [mg EPS]⁻¹), glucose (19.9 µg [mg EPS]⁻¹) and mannose (31.3 µg [mg EPS]⁻¹). The sugar composition of the EPS consisted of hexoses (glucose, 25.4%, and galactose, 24.5%) and pentoses (mannose, 39.9%, and arabinose, 10.1%). The molar percentage of mannose was the highest, followed by glucose, galactose and arabinose. Glucose, galactose and fucose were previously detected as the major sugars in the EPS produced by *V. parahaemolyticus* (Enos-Berlage and McCarter 2000) grown in heart infusion medium, with negligible amounts ( < 1%) of mannose and arabinose. In the present study, the sugars mannose and arabinose were obtained as the major sugars present in the EPS; this could possibly be due to the different culture medium used for the growth in present study; Zobell 2216 provides different carbon and nutrient sources than heart infusion medium, and this can determine the quality and quantity of polysaccharide formation (Cerning et al. 1994; Nourani et al. 1998). Heart infusion medium contains 1% beef heart, 1% tryptose and 0.5% sodium chloride (w/v), whereas, Zobell 2216 medium is composed of 0.5% peptone, 0.1% yeast extract and all essential minerals (ferric citrate 0.01%; sodium chloride, 1.945%; magnesium chloride, 0.88%; sodium sulphate, 0.324%; calcium chloride, 0.18%;

![Figure 2. Representative PXRD profile of EPS isolated from *V. parahaemolyticus*.](image)

![Figure 3. GCMS profile of EPS obtained from *V. parahaemolyticus*.](image)
potassium chloride, 0.055%; sodium bicarbonate, 0.016%; potassium bromide, 0.008%; strontium chloride, 34 ppm; boric acid, 22 ppm; sodium silicate, 4 ppm; sodium fluoride, 2.4 ppm; ammonium nitrate 1.6, ppm and disodium phosphate, 8 ppm; (w/v)), closely duplicating the mineral composition of seawater. Peptone and yeast extract (in the Zobell medium) provide nitrogen, vitamins and minerals while the high salt content simulates seawater. Heart infusion broth is a nonselective, general-purpose medium used in the isolation and cultivation of a wide range of microorganisms, from a variety of clinical specimens and nutritionally fastidious microorganisms (Elliott et al. 1995; Atlas and Parks, 1997). Zobell 2216 medium contains most of the nutrients necessary for the growth of marine bacteria. It is specifically used for cultivating heterotrophic marine bacteria (Kwon et al. 2002). The EPS produced by V. harveyi strain VB23 also contained the same monosaccharide constituents along with rhamnose, fucose, ribose, and xylose (Bramhachari and Dubey, 2006).

MALDI TOF–TOF mass spectrometric analysis revealed a series of masses (m/z) corresponding to pentose and hexose sugars (150 and 180, respectively) individually or combined as disaccharides (ie 2 pentoses, ~300; 1 pentose + 1 hexose, ~330 and 2 hexoses, ~360) in midrange linear mode (Supplementary Figure S5a [Supplementary material is available via a multimedia link on the online article webpage]). The positive ion reflector mode exhibited a higher m/z range attributed to oligosaccharides comprised of hexose and pentose moieties linked in different combinations (Supplementary Figure S5b [Supplementary material is available via a multimedia link on the online article webpage]). Positive ion linear mode has been reported to be suitable for the analysis of oligomers, whereas reflector mode has been recommended for polysaccharide analysis (Mishra et al. 2011). This work contains the first report of a MALDI TOF–TOF analysis of bacterial EPS, whereas MALDI TOF mass spectroscopy for a bacterial EPS was recently reported (Gauri et al. 2009).

**NMR and SEM**

The $^1$HNMR spectrum of the EPS revealed characteristic chemical shifts (ppm) and corresponding functional groups (Figure 4). The 4.8 ppm shift is attributed to the $\beta$-anomeric carbons of hexose or pentose (Mishra et al. 2011), and the chemical shift of the functional group $R_2CHOR$ was observed at 3.2–4.3 ppm. A stretching of the N–H group was observed at 1.3 ppm and for alkanes at 0.8–1.2 ppm (CH$_3$ group).

Figure 4. Proton nuclear magnetic resonance ($^1$HNMR) spectrum of EPS extracted from V. parahaemolyticus.

Figure 5. Thermogravimetric (a) and DSC (b) thermogram of EPS obtained from V. parahaemolyticus at a heating rate of 10°C.
and 1.1–1.5 ppm (CH$_2$ group). The spectrum at 2.0 ppm corresponded to the acetyl amines of hexose or pentose, and the peak at 2.6 ppm substantiates the presence of the functional group H–C–COOH. The presence of acetyl groups rendered the EPS somewhat hydrophobic, which contributed to their emulsifying capacity (Mata et al. 2006), as observed in this study.

It was clear from the SEM image (Supplementary Figure S6 [Supplementary material is available via a multimedia link on the online article webpage]) that the exopolymer was compact in nature with small pores. Compactness with porosity was also observed in the EPS extracted from an *Azotobacter* sp. (Gauri et al. 2009).

**TG and DSC analysis**

The thermal behaviour of EPS plays an important role in their commercial exploitation (Marinho-Soriano and Bourret 2005). The thermal degradation of the EPS occurred in two steps: 13% of the EPS was degraded on heating to 160°C, followed by a second phase of depolymerisation (33%) occurring at 380°C (Figure 5a). The transition from an amorphous solid to a crystalline solid was an exothermic process, and differential scanning calorimetric analysis showed a significant thermal transition in the EPS (Figure 5b). The DSC thermogram showed a transition characteristic of an exopolymer, with a crystallisation temperature ($T_c$) of 107.35°C (onset temperature 102.49°C) and a latent energy of crystallisation of 1542.00 mJ g$^{-1}$, followed by a melting transition. The melting temperature ($T_m$) of the EPS was determined to be 249.60°C (onset temperature 274.51°C), with a 51.94 mJ g$^{-1}$ latent energy of melting. The activation energy (for the $n$th-order reaction) of the first transition (crystallisation) was 42.68 ± 0.51 kJ mol$^{-1}$; for the second transition (melting), it was 187.68 ± 0.79 kJ mol$^{-1}$, which is lower than that of EPS isolated from cyanobacteria (Parikh and Madamwar 2006). The extracted EPS was found to be thermostable up to 250°C, thus making it a promising additive for industrial applications. A similar thermogram was observed with EPS extracted from *Paenibacillus pabuli* (Pooja and Chandra 2009).

**Rheological studies**

The viscosity ($\eta$) of the EPS decreased concomitantly with shear rate ($\gamma$), showing a pseudoplastic rheological behaviour (Figure 6a). The pseudoplasticity was more profound up to a shear rate of 250–300 s$^{-1}$; thereafter, Newtonian behaviour was observed. The EPS showed shear thinning behaviour at both low and neutral pH, making them a promising additive for the food industry, as they might provide suspension and sensory qualities in food products (Enrı́quez et al. 1989). Shear thinning is mainly due to the breakdown of structural units in EPS by hydrodynamic forces, generated during shear. Similarly, viscosity decreased concurrently with temperature at both pH values (Figure 6b), following a pattern similar to that of the EPS extracted from *V. alginolyticus* (Muralidharan and Jayachandran 2003). The EPS extracted from *V. parahaemolyticus* showed a rheology independent of pH, whereas in the EPS produced by several bacterial species, high viscosity was observed at low pH (Béjar et al. 1998; Gauri et al. 2009).

**Conclusions**

The biopolymer produced by the biofilm-forming marine bacterium *V. parahaemolyticus* was amorphous (CI$_{xrd}$ 0.092) and thermostable up to 250°C. The FTIR spectrum of the EPS revealed the presence of aliphatic

![Figure 6. Effect of (a) shear rate and (b) temperature on the viscosity of EPS isolated from *V. parahaemolyticus*.](image-url)
methyl groups, primary amines, halide groups, uronic acid and saccharides (galactose and mannose). Four monosaccharides (arabinose, galactose, glucose and mannose) were detected by GCMS. The EPS were also characterised by advanced analytical methods, including MALDI-TOF–TOF analysis, which constitutes the first report of this application for EPS analysis. The EPS showed a pH-independent pseudoplastic rheology and stable emulsifying activity, making the EPS a very promising candidate for commercial exploitation. However, further work is required to determine the suitability of this EPS for biotechnological applications.

Acknowledgements
The financial support received from CSIR (NWP-0018), Government of India is thankfully acknowledged. The Analytical Science Discipline of the Institute is duly acknowledged for helping in analysis.

References
Aldred N, Clare AS. 2008. The adhesive strategies of cyprids and development of barnacle resistant marine coatings. Biofouling 24:351–363.
Andersson S, Dalhammar G, Land CJ, Rajarao GK. 2009. Characterization of extracellular polymeric substances from denitrifying organism Comamonas denitrificans. Appl Microbiol Biotechnol 82:535–543.
Atlas RM, Parks LC. 1997. Handbook of microbiological media. 2nd ed. Boca Raton (FL): CRC Press, Inc. pp. 1097.
Bejar V, Llamas I, Calvo C, Quesada E. 1998. Characterization of exopolysaccharides produced by 19 halophilic strains of the species Halomonas earthalina. J Biotechnol 61:135–141.
Bramhachari PV, Dubey SK. 2006. Isolation and characterization of exopolysaccharide produced by Vibrio harveyi strain VB23. Lett Appl Microbiol 43:571–577.
Bramhachari PV, Kavi-kishor PB, Ramadevi R, Kumar R, Rao BR, Dubey SK. 2007. Isolation and characterization of mucous exopolysaccharide (EPS) produced by Vibrio furnissii strain VB0S3. J Microbiol Biotechnol 17:44–51.
Cerning J, Renard CMGC, Thibault JF. 1994. Carbon source requirements for EPS production by Lactobacillus casei CG11 and partial structure analysis of the polymer. Appl Environ Microbiol 60:3914–3919.
Checa FM, Toledo FL, Mabrouki KE, Quesada E, Calvo C. 2007. Characteristics of bioemulsifier V2-7 synthesized in culture media added of hydrocarbons: chemical composi-
Chie Z, Su CD, Lu WD. 2007. A new exopolysaccharide produced by marine Cyanothoece sp. 113. Bioresearch Technol 98:1329–1332.
de Carvalho CCCCR, Fernandes P. 2010. Production of metabolites as bacterial responses to the marine environment. Mar Drugs 8:705–727.
Dickson JS. 2010. Quorum sensing and bacterial biofilms. Nat Prod Rep 27:343–369.
Dobretsov S, Teplitski M, Paul V. 2009. Quorum sensing in the marine environment and its relationship to biofouling [mini-review]. Biofouling 25:413–427.
Dobois M, Giles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356.
Elliott EL, Kaysner CA, Jackson L, Tamplin ML. 1995. V. cholera, V. parahaemolyticus, V. vulnificus and other Vibrio spp. In: Tomlinson LA, editor, USFDA Bacteriological analytical manual. 8th ed. Gaithersburg (MD): AOAC International. p. 9.01–9.27.
Enos-Berlage JL, McCarter LL. 2000. Relation of capsular polysaccharide production and colonial cell organization to colony morphology in Vibrio parahaemolyticus. J Bacteriol 182:5513–5520.
Enriquez LG, Hwang JW, Hong GP, Bati NA, Flick GJ. 1989. Plant and microbial food gums. In: Charalambons G, Doxastakis G, editors. Food emulsifiers: chemistry, technology, functional properties and applications. New York: Elsevier. p. 335–416.
Flemming HC, Neu TR, Wozniak DJ. 2007. The EPS matrix: the “house of biofilm cells”. J Bacteriol 189:7945–7947.
Freitas F, Alves VD, Pais J, Costa N, Oliveira C, Mafra L, Hilliou L, Oliveira R, Reis MA. 2009. Characterization of an extracellular polysaccharide produced by a Pseudomonas strain grown on glycerol. Bioresearch Technol 100:859–865.
Gauri SS, Mandal SM, Mondal KC, Dey S, Pati BR. 2009. Enhanced production and partial characterization of an extracellular polysaccharide from newly isolated Azoto bacter sp. SSBB1. Bioresearch Technol 100:4240–4243.
Iyer A, Mody K, Jha B. 2005. Characterization of an exopolysaccharide produced by a marine Enterobacter cloacae. Indian J Exp Biol 43:467–471.
Khattar JIS, Singh DP, Jindal N, Kaur N, Singh Y, Rahi P, Gulati A. 2010. Isolation and characterization of exopolysaccharides produced by the cyanobacterium Limnothrix redekii PUPCCC 116. Appl Biochem Biotechnol 162:1327–1338.
Kwon KK, Lee HS, Jung SY, Yim JH, Lee JH, Lee HK. 2002. Isolation and identification of biofilm-forming marine bacteria on glass surface in Dae-Ho Dike, Korea. J Microbiol 40:260–266.
Marinho-Soriano E, Bourtet E. 2005. Polysaccharides from the red seaweed Gracilaria dura (Gracilariales, Rhodophyta). Bioresearch Technol 96:379–382.
Mata JA, Bejar V, Llamas I, Arias S, Bressollier P, Tallon R, Kwon KK, Lee HS, Jung SY, Yim JH, Lee JH, Lee HK. 2009. Enhanced production and partial characterization of mucous extracellular polysaccharide produced by Vibrio furnissii strain grown on glycerol. Bioresearch Technol 100:859–865.
Mehta GK, Meena R, Prasad K, Ganesan M, Siddhanta AK. 2010. Preparation of galactans from Gracilaria dasyliis and Gracilaria salicornia (Gracilariales, Rhodophyta) of Indian waters. J Appl Phycol 22:623–627.
Mishra A, Jha B. 2009. Isolation and characterization of extracellular polymeric substances produced by micro-algae Dunaliella salina under salt stress. Bioresearch Technol 100:3382–3386.
Mishra A, Kavita K, Jha B. 2011. Characterization of extracellular polymeric substances produced by micro-algae Dunaliella salina. Carbohydr Polym 83:852–857.
Muralidharan J, Jayachandran S. 2003. Physicochemical analyses of the exopolysaccharides produced by a marine biofouling bacterium, *Vibrio alginolyticus*. Process Biochem 38:841–847.

Nourani GL, Blondeau K, Simonet JM. 1998. Influence extraction and purification of EPS from *Klebsiella pneumoniae*. J Microbiol Methods 9:211–220.

Parikh A, Madamwar D. 2006. Partial characterization of extracellular polysaccharides from cyanobacteria. Bioresour Technol 97:1822–1827.

Pongjanyakul T, Puttipipatkhachorn S. 2007. Xanthan-alginate composite gel beads: molecular interaction and *in vitro* characterization. Int J Pharmacol 331:61–71.

Pooja KP, Chandra TS. 2009. Production and partial characterization of a novel capsular polysaccharide KP-EPS produced by *Paenibacillus pabuli* strain ATSKP. World J Microbiol Biotechnol 25:835–841.

Ricou P, Pinel E, Juhasz N. 2005. Temperature experiments for improved accuracy in the calculation of polyamide-11 crystallinity by X-ray diffraction. Advances in X-ray analysis, volume 48. International Centre for Diffraction Data. Pennsylvania: International Centre for Diffraction Data. p. 170–175.

Satpute SK, Banat IM, Dhakephalkar PK, Banpurkar AG, Chopade BA. 2010. Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. Biotechnol Adv 28:436–450.

Subramanian BS, Yan S, Tyagi RD, Surampalli RY. 2010. Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering. Water Res 44:2253–2266.

Vu B, Chen M, Crawford RJ, Ivanova EP. 2009. Bacterial extracellular polysaccharides involved in biofilm formation. Molecules 14:2535–2554.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703.

Yildiz FH, Visick KL. 2008. *Vibrio* biofilms: so much the same yet so different. Trends Microbiol 17:109–118.

Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences. J Comput Biol 7:203–214.