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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