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Characterisation and anti-biofilm activity of extracellular polymeric substances from *Oceanobacillus iheyensis*

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ABSTRACT

Oceanobacillus iheyensis BK6, isolated from a marine natural biofilm, produced approximately 400 mg L^{-1} extracellular polymeric substances (EPS). FTIR analysis of the EPS revealed different functional groups (halide groups, uronic acid and saccharides). The GCMS showed that the extracellular polysacchrides comprised of mannose (47.78%), glucose (29.71%) and arabinose (22.46%). The molecular mass of the EPS was about 2.14×10^6 Da. It was thermally stable and showed pseudoplastic rheology and emulsifying activity (66.47%). The EPS exhibited antibiofilm activity against a pathogenic strain of *Staphylococcus aureus*. This is the first report on the characterisation of EPS from the genus *Oceanobacillus*. The high viscosity, emulsifying properties and antibiofilm activity of EPS make it suitable for potential pharmaceutical and industrial applications.

1. Introduction

Bacterial biofilms are communities of microorganisms that live in a self-produced matrix composed of extracellular polymeric substances (EPS) (Costerton, Stewart, & Greenberg, 1999; Flemming & Wingender, 2010). Biofilms are commonly present in natural (sea, rivers, sub-merged rocks etc.), medical (plaque and medical implants) and engineered systems such as pipelines of waters, sewage, offshore oil and gas industry (McDougald, Rice, Barraud, Steinberg, & Kjelleberg, 2012). EPS mainly consists of polysaccharides, proteins, extracellular DNA and lipids (Flemming & Wingender, 2010). In recent years, there has been an increased interest in exploring valuable EPS due to its various industrial applications, and attention on EPS-producing biofilm-forming bacteria has been greatly enhanced. The wide structural, physical and rheological diversity and other unique properties of EPS produced by biofilm-forming bacteria make it industrially and biotechnologically important (Vu, Chen, Crawford, & Ivanova, 2009). The unique biophysicochemical properties of bacterial EPS make it widely useful in the food industry as viscosifying, stabilising and emulsifying agents (Liu et al., 2010; Mishra & Jha, 2013). In addition, EPS could be used as bioflocculants, bioabsorbents, encapsulating materials, heavy metal removing agents, drug delivery agents, ion exchange resins, a natural immunomodulator, and antioxidant and antibiofilm agents (Ismail & Nampoothiri, 2010; Kanmani, Yuvaraj, Paari, Pattukumar, & Arul, 2011; Liu et al., 2010).

Biofilm formation is an important factor in chronic and recurrent infections of pathogenic bacteria (Kim, Oh, & Kim, 2009). Therefore, most of the research work has focused on identifying alternative ways of preventing biofilm formation for the complete eradication of pathogenic bacteria (Kanmani et al., 2011; Kim et al., 2009). Kanmani et al. (2011) reported that EPS from *Streptococcus phocae* PI80 have the ability to inhibit the biofilm formation by pathogenic bacterium.

Although a number of reports are available for EPS from different bacteria and habitats, the marine environment, which supports a rich biodiversity of marine bacteria, remains unexplored (Kavita, Mishra, & Jha, 2011; Kavita, Mishra, & Jha, 2013). In this study, EPS from a biofilm-forming marine bacterium, *O. iheyensis* strain BK6, was extracted and studied with respect to its biophysicochemical analysis using a battery of techniques. Some important properties, such as its antibiofilm potential against the pathogenic bacterium *S. aureus*, thermo-stability, rheology and emulsifying activity, were investigated for their future industrial applications. This is the first report on detail characterisation of EPS from any species of *Oceanobacillus*, so far.

2. Materials and methods

2.1. Isolation and identification of the marine bacterium

Bacteria were isolated from natural biofilm collected from the coastal region of Sikka, India (latitude N 22° 25′ 98.0″ and

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longitude E 69° 49′ 3.85″), using Zobell marine medium (Kavita et al., 2011). A bacterial isolate BK6 was selected on the basis of its maximum EPS-producing ability, as determined by quantification of EPS. The selected isolate was identified by 16S rRNA gene sequence homology. Whole cell fatty acid profiling was performed with a Microbial Identification System coupled with gas chromatography (GC system–6850, Agilent technologies, USA) (Gontia, Kavita, Schmid, Hartmann, & Jha, 2011), using the RTSBA6 6.10 database. The 16S rRNA gene was 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′) (Weisburg, Barns, Pelletier, & Lane, 1991), sequenced, analysed and submitted to GenBank (NCBI).

2.2. Production and purification of EPS

Released EPS was extracted from planktonic culture of *O. iheyensis* strain BK6 using an optimised procedure (Kavita et al., 2011). Briefly, Zobell marine broth (500 mL) was inoculated with 2% (v/v) overnight culture ($OD_{600\ nm}$ = 0.6) and incubated for two days at 30 °C on a rotator shaker (180 rpm). The bacterial culture was centrifuged at 15,000 *g* for 20 min at 4 °C. The obtained supernatant was filtered twice and concentrated to 100 mL using a rotary evaporator (Buchi, Switzerland) for 8–10 h. Two volume cold isopropanol was gradually added to the concentrated supernatant and retained for precipitation at 4 °C for 16 h. The precipitated EPS was washed, dissolved in Milli-Q water and kept for dialysis (12,000 Da cut off dialysis tubing, Sigma, USA). The dialysed EPS was lyophilised, quantified by weighing and stored in a dry place for further analyses. The lyophilised EPS was analysed to determine the total sugar, protein and uronic acid contents (Bradford, 1976; Knutson & Jeanes, 1968; Morris, 1948).

2.3. Characterisation of EPS

2.3.1. Determination of mass

The molecular weight of EPS was determined by gel permeation chromatography (GPC; 7.8 mm ID × 300 mm stainless steel, Model Alliance 2695, Waters, USA) equipped with a guard column. The EPS solution (0.1%, w/v) was prepared in Milli-Q water and approximately 50 μl was subjected to GPC column Ultrahydrogel-120 and Ultrahydrogel-500 at 30 °C. Dextran (molecular weight, 5200–668,000 kDa; PSS, USA) was used as the standard, and elution was monitored by a refractive index detector (Jain, Mody, Mishra, & Jha, 2012).

2.3.2. Sugar composition

The monosaccharide composition of EPS was evaluated by gas chromatography mass spectroscopy (GC–MS). The EPS was converted to an alditol-acetate derivative and subjected to GC–MS analysis (Shimadzu, QP-2010) using the previously described method (Kavita et al., 2013). Briefly, purified EPS (50 mg) was hydrolysed with 2 M H_2SO_4 (5 mL) at 100 °C for 6 h in a sealed glass tube. The hydrolysed acidic sample was neutralised by $BaCO_3$, filtered and concentrated to 5 mL. Thereafter, 2 ml of $NaBH_4$ (15 mg mL^{-1} in distilled water) was added for reduction purposes, and the mixture was kept overnight (12 h at 25 °C). The reduced solution was subjected to a cation exchange resin column (rate = 5 mL min^{-1}), which was further concentrated using methanol. For this, 10 mL of methanol was added and concentrated to 2 ml using rotary evaporator. This step was repeated thrice and thereafter the resulting 2 mL sample-solution was kept for drying in desiccators (16 h). An equal volume (1 mL each) of pyridine and acetic anhydride was added into the dried sample and refluxed at 100 °C for 30 min; a yellow-coloured product was obtained. This product was added to ice water and extracted with ethyl acetate. The solution was subjected to subsequent washing with Milli-Q water, saturated Na_2CO_3 and $CuSO_4$ in order to ensure the solution was free from excess acetic anhydride and pyridine. The moisture was removed by adding anhydrous Na_2SO_4. The moisture-free organic layer was separated and kept under vacuum desiccators overnight. Finally, 0.1 mg dried powder was dissolved in 5 mL dichloromethane, filtered and subjected to GC–MS analysis.

2.3.3. Functional groups analysis

Functional groups of purified EPS were determined using Fourier transformed infrared (FTIR) spectroscopy analysis. The pellet was prepared by pressing the mixture of EPS and KBr (1:100) into a mould and the FTIR spectrum was acquired in the 4000–400 cm^{-1} region with a resolution of 4 cm^{-1} using a GX FTIR system (PerkinElmer, USA) (Kavita et al., 2013; Mishra, Kavita, & Jha, 2011).

2.3.4. Elemental analysis

Energy dispersive X-ray spectroscopy (EDX) was used for the elemental analysis of EPS. The EPS (5 mg) was attached on a stub and analysed by SEM (scanning electron microscope) -EDX (SEM-EDX, Oxford Instruments, UK) (Jain et al., 2012). The X-rays emitted by matter revealed the weight and atomic percentage of the different elements present in the sample.

2.4. Thermal properties of EPS

The thermal properties of EPS were determined by using thermal gravimetric (TG) and diffraction scanning colorimetric (DSC) analysis. TG and DSC analysis of purified EPS (5 mg) was performed on a Mettler Toledo TGA/SDTA System (Greifensee, Switzerland), with energy level scanning in the range of 30–600 °C (temperature gradient 10 °C min^{-1}) under a nitrogen atmosphere. Thermograms of TG and DSC analyses were obtained by plotting the weight (percentage) and heat flow, respectively, vs. temperature (Kavita et al., 2013).

2.5. Visual appearance and nature of EPS

The visual appearance of EPS was evaluated using SEM and atomic force microscopy (AFM). The surface morphology of EPS was visualised by using SEM (LEO series VP1430, Germany) with an accelerating voltage of 20 kV (Kavita et al., 2011). Surface topography was determined using AFM (Ntegra-Aura, NT-MDT, Moscow, Russia) in tapping mode. The topographic AFM images 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 such as surface bearing index (S_{bi}), core fluid retention index (S_{ci}) and valley fluid retention index (S_{vi}) (Kavita et al., 2013).

2.6. Applications of EPS

2.6.1. In vitro antibiofilm activity

The antibiofilm activity was determined using the modified method, described previously (Andersson, Dalhammar, Land, & Rajarao, 2009). A multidrug-resistant clinical isolate of *S. aureus* was kindly provided by the Department of Microbiology, Government Medical College, Bhavnagar, Gujarat, India, grown at 37 °C in nutrient broth. The culture, grown overnight, was diluted to 0.1 OD with nutrient broth at 620 nm and then added to a 96-well polystyrene microtiter plate together with different concentrations of EPS (25–200 μg mL^{-1}). The plate was incubated for 24 h (100 rpm, 37 °C) and a biofilm developed by strain in the absence of EPS was used as the control. The bacterial growth was measured by colony forming unit (CFU) method in order to examine the effect of EPS on growth. Wells were washed, dried and stained with 1% crystal violet, and excess dye was washed off. Thereafter, 200 μL

96% ethanol was added and absorbance was measured at 590 nm on a spectrophotometer (SpectraMax plus 384, Molecular Devices, USA) (Andersson et al., 2009). The experiment was performed in five replicates. Statistical analysis was performed using Sigmaplot 12. All values were expressed in terms of mean ± SD. A one-way ANOVA followed by a Dunnett's post-test was applied for comparison of the test and control, with a P value of <0.05 considered as significant.

2.6.2. Emulsifying activity

The emulsifying activity of lyophilised EPS was determined as described previously (Bramhachari & Dubey, 2006). In brief, purified EPS solution (2 mg mL^{-1} in deionised water) was heated at 100 °C for 15 min, followed by cooling (25 °C), and the volume was made upto 2 mL using phosphate-buffered saline (PBS). One millilitre of hexadecane was added, vortexed for 1 min and the absorbance was measured after 30 min at 540 nm. The emulsifying activity was calculated in terms of percentage decline in the absorbance at different time intervals.

2.6.3. Rheological activity

Lyophilised EPS was dissolved in distilled water (0.4%, w/v) and a dynamic rheological measurement was carried out on the rheometer (Anton Paar Physica MCR 301, USA) using parallel plate PP50/P-PTD200 geometry (50 mm diameter; 0.1 mm gap) at different temperatures, applied shear rate and pH, with a 1 mL sample volume (Kavita et al., 2013). Measurements were carried out immediately after placing the sample on the plate and the outer surface of the samples were covered with silicon oil to avoid loss of moisture due to evaporation at higher temperature (Kavita et al., 2013). The viscosity was measured at 25 °C with different shear rates (50–950 s^{-1}) and pH (3.0 and 7.0). The influence of temperature (10–60 °C) was also analysed at both pH values. All experiments were carried out in triplicate.

2.6.4. Reduction in surface tension

Surface tension reduction ability of EPS was measured by a du Nouy ring tensiometer at 30 °C (Data Physics, Germany). The bacterial strain was grown in Zobell marine broth (100 mL) and cell-free supernatant was aspirated at different time intervals (12, 24 and 48 h), and the surface tension was measured. The surface tension of distilled water and Zobell marine broth was used as control (Jain, Mody, Joshi, Mishra, & Jha, 2013).

3. Results and discussion

3.1. Bacterial identification

A total of twenty-three bacteria were isolated from a natural marine biofilm using Zobell marine agar. Of these, one strain producing maximum EPS was selected for further study. The 16S rRNA gene sequence of the isolate (GenBank: JX182989) showed 99% sequence similarity with *O. iheyensis*. Thus the selected bacterial isolate was identified as *O. iheyensis* based on the 16S rRNA gene sequence homology. The whole cell fatty acid profiling of the isolate determined the presence of 15:0 anteiso, 15:0 iso, 14:0 iso, 16:0 iso and 17:0 anteiso as the major characteristic fatty acids (Table S1), similar to the previous report on *O. iheyensis* (Lu, Nogi, & Takami, 2001).

3.2. Extraction of EPS

Maximum EPS (396 mg L^{-1}) was produced after 48 h in Zobell marine medium by *O. iheyensis*. It comprised of about 70% sugars, 10% protein and 5% uronic acid. The EPS production was significantly greater than that reported for other marine bacteria such as *Bacillus* strain B3-15 (165 mg L^{-1}) (Maugeri et al., 2002), *Vibrio parahaemolytics* (58.98 mg L^{-1}) (Kavita et al., 2011) and *V. fortis* (134 mg L^{-1}) (Kavita et al., 2013). However, it is comparable to the EPS of *V. campbellii* (400 mg L^{-1}) (Kavita et al., 2013).

3.3. Characterisation of extracellular polymeric substances

3.3.1. Molecular mass and elemental analysis

The GPC analysis of EPS showed a single peak at approximately 2.14×10^6 Da with a 3.10 polydispersity and 16.1 min retention time (Fig. S1). High molecular weight exopolymers have also been reported from *Bacillus* strain B3-15 (6×10^5 kDa), *Idiomarina* spp. (1.5×10^4 to 1.5×10^6 kDa) and *Klebsiella sp*. (3.7×10 Da) (Jain et al., 2012; Mancuso Nichols, Garon, Bowman, Raguénès, & Guezennec, 2004; Manzoni & Rollini, 2001). The high molecular weight exopolymers are associated with production of stable emulsions (Bonilla, Olivaro, Corona, Vazquez, & Soubes, 2005) and it was reported that they have great potential to be used as biosurfactants (bioemulsifiers) (Banat et al., 2010). The elemental composition of the EPS was determined by SEM-EDX and the analysis revealed the presence of different elements (Table S2). In addition to common elements (like C, N, O, Mg and Na), sulphate and phosphate residues were also observed in the EPS produced by *O. iheyensis*. Sulphated EPS is also reported from the marine bacterium, *Pseudoalteromonas* strain CAM036 (Nwodo & Okoh, 2012).

3.3.2. Functional group analysis

The functional group of the EPS was determined by FTIR and NMR spectroscopy. The FTIR analysis (Fig. 1) revealed a broad stretched peak at 3402 cm^{-1} (range 3600–3200 cm^{-1}), corresponding to the hydroxyl group (Kavita et al., 2013; Nwodo & Okoh, 2012). A weak absorption at 2961 cm^{-1} was assigned to an asymmetrical C—H stretching vibration of the aliphatic CH_2 group, which represents the presence of organic substances like sugars, proteins etc. (Kavita et al., 2013). The peak observed at approximately 2359 cm^{-1} was either due to CO_2 adsorption or may be from amine group (Ahluwalia & Goyal, 2005). Presence of an asymmetric medium stretching peak at 1647 cm^{-1} may corresponded to the ring stretching of mannose or galactose (Freitas et al., 2009a). Another peak at 1413 cm^{-1} could be attributed to the symmetric stretching of the —COO^- group (Kavita et al., 2011). The absorption peaks ranging from 1000–1200 cm^{-1} were designated to C—O—C and C—O, which indicate the occurrence of carbohydrates (Freitas et al., 2009a; Kavita et al., 2011). A peak at 1103 cm^{-1} (1000–1125 cm^{-1} range) may be attributed to O-acetyl ester linkage bond of uronic acid (Bramhachari & Dubey, 2006). The absorption peak at approximately 616 cm^{-1} (690–515 cm^{-1} range) corresponded to stretching of alkyl-halides (Kavita et al., 2011). The FTIR spectrum determined the presence of halide groups, uronic acid and saccharides such as mannose. The presence of diverse functional groups may impart different applications. Water solubility of EPS is attributed to the presence of hydroxyl group (Karbowiak, Ferret, Debeaufort, Voilley, & Cayot, 2011) while the presence of aliphatic CH_2 groups are responsible for hydrophobic nature. The hydrophilic and hydrophobic nature of EPS makes it suitable as emulsifying agent.

3.3.3. Sugar composition

The GC–MS analysis of sugar component of EPS showed the presence of three monosaccharides, namely mannose (47.78 mole%), glucose (29.71 mole%) and arabinose (22.46 mole%). Mannose as a major constituent of EPS is also reported from another bacterium *V. parahaemolyticus*, isolated from natural biofilm (Kavita et al., 2011). Mannose is the only sugar in the EPS of the biofilm- forming bacterium *Thermococcus litoralis* (Rinker & Kelly, 1996). To check

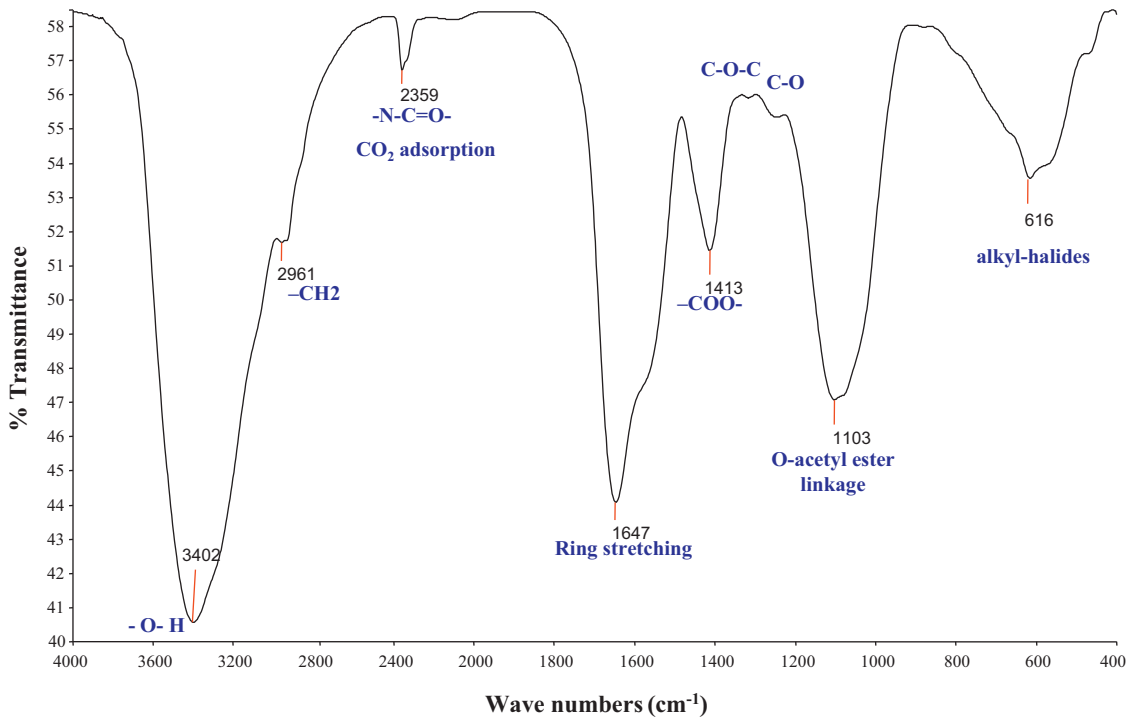

Fig. 1. FTIR spectrum of EPS produced by *O. iheyensis* strain BK6.

whether the medium contributed to mannose in the EPS, an additional experiment was conducted to purify mannose containing polysaccharide from growth medium alone. Zobell marine broth (500 mL) was used to extract polysaccharides exactly the way it was done for EPS extraction. We could not get any detectable mannose. In a similar experiment mannose polysaccharide could not be detected even in the medium (Terrific broth – 2.4% yeast extract) with much higher yeast extract (Sandal et al., 2011). When two different strains of *Streptococcus thermophilus* (*S. thermophilus* Sfi20 and *S. thermophilus* LY03) were grown and subcultured in MRS medium containg 1.2% yeast extract, mannose could also be detected. However, more mannose was isolated from the medium in the case of *S.thermophilus* LY03 than in the case of *S. thermophilus* Sfi20 (Degeest, Vaningelgem, Laws, & De Vuyst, 2001). Earlier, Rinker and Kelly (1996) have detected the presence mannose in the EPS of *Thermococcus litoralis* grown in defined medium without yeast extract. We were not able to detect mannose in the medium only, however, the contribution of yeast extract cannot be completely ruled out.

3.4. Thermal properties

The thermal stability of EPS is an important characteristic for its commercial exploitation (Marinho-Soriano & Bourret, 2005). The TG analysis showed that EPS degrades in two steps (Fig. 2A). In the first step, weight loss (11.2%) was observed up to 130 °C, due to the loss of moisture molecules in the biopolymer (Jain et al., 2012). In the second step, depolymerisation occurred up to 380 °C and a weight loss of about 40% was observed. The thermal transition of the EPS was studied by differential scanning calorimetric analysis (Fig. 2B). The transition of amorphous to crystalline solid was a heat-generating (exothermic) process and the DSC thermogram exhibited the crystallisation process at a temperature (T_C) of about 83 °C. Thereafter the melting transition started from 176 °C. The physical nature of EPS may be considered as partially crystalline polymer containing both crystalline and amorphous regions. The crystalline EPSs are generally resistant to solvents and chemicals compared to non-crystalline (amorphous) EPSs. The thermal stability of the EPS of *O. iheyensis* makes it a promising additive as thickening and gelling agent in food industries.

3.5. Visual appearance and nature

SEM and AFM are widely used to study the morphological features of polymers. Scanning electron micrography shows the compact nature of EPS (Fig. S2). It was found that compact structure is a characteristic of a plasticised film (Wang et al., 2010). In recent years, AFM has been extensively used to study the morphological characteristics of different polymers. The AFM analysis of the EPS of *O. iheyensis* showing the height distribution profile is depicted in Fig. S3. The average surface roughness of 60.61 nm, roughness root mean square (R_q) (71.02), surface skewness (R_{sk}) (0.055), coefficient of kurtosis (R_{ku}) (2.01)., area root mean square slope (A_q) (0.112) and functional indices such as surface bearing index (S_{bi}) (0.67), core fluid retention index (S_{ci}) (1.53) and valley fluid retention index (S_{vi}) (0.07) were calculated. The functional indices exhibit physical characteristics of the polymer (Ţălu, 2013). The S_{bi} and S_{ci} indicate a good bearing property and fluid retention, respectively while S_{vi} indicates a good fluid retention in the valley zone. The AFM topograph suggests tightly packed molecules with a reticulate shape which impart a pseudoplastic behaviour of the EPS (Ahmed, Wang, Anjum, Ahmad, & Khan, 2013) as detected by rheology studies.

3.6. Potential applications of EPS

3.6.1. Antibiofilm activity

A biofilm formation assay with varying concentration of EPS was performed in order to study the antibiofilm potential of the EPS against a pathogenic strain of *S. aureus*. Biofilm formation in *S. aureus* decreased significantly ($P < 0.05$) with increasing concentrations of EPS (Fig. 3). The maximum inhibition (62.3%) was observed when 175 μg mL^{-1} of EPS was used. However, no further increase in inhibition was observed at higher EPS concentration

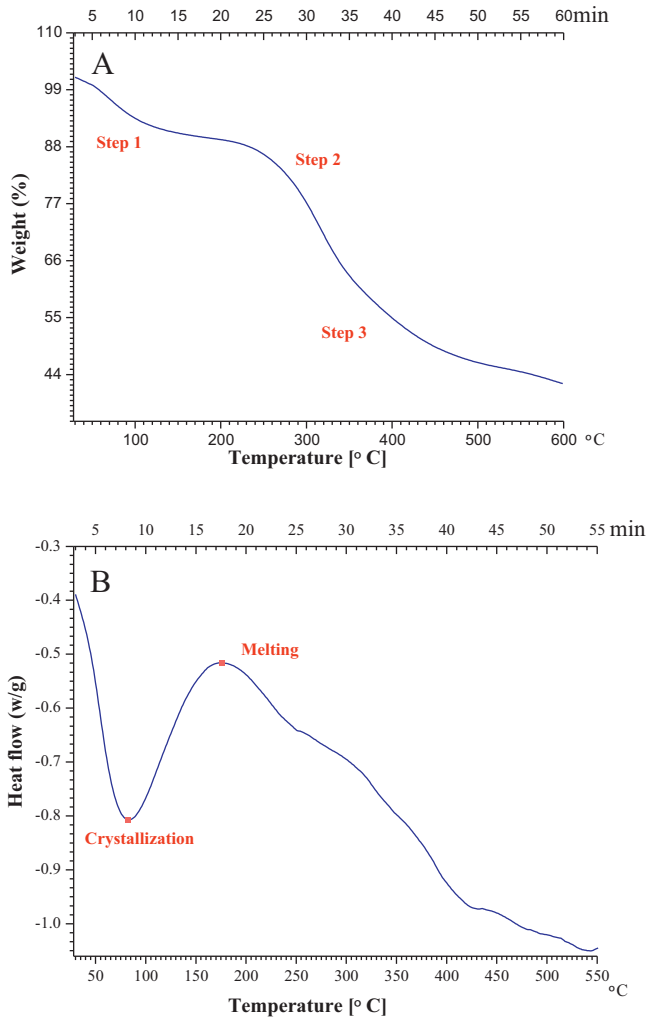

Fig. 2. Thermogravimetric (A) and DSC (B) thermogram of EPS extracted from *O. iheyensis*.

(200 μg mL^{-1}) (Fig. 3). The percentage biofilm inhibition (62.3%) by EPS of *O. iheyensis* is greater than those reported for EPSs from *S. phocae* PI80 (51%) (Kanmani et al., 2011) and *Enterococcus faecium* MC13 (48%) (Kanmani et al., 2013) against *S. aureus*. It is important to note that the magnitude of inhibition reported in this study could be achieved using a much lower concentration (175 μg mL^{-1}) than the concentration (1 mg mL^{-1}) used for the EPSs of *S. phocae* PI80 and *E. faecium* MC13 (Kanmani et al., 2011, 2013).

The CFU of cultures with or without EPS was counted to determine whether EPS adversely affected cell growth, no significant difference between treatment and control was observed (data not shown). This indicated that the inhibition of biofilm in *S. aureus* is not due to the toxic effect of the EPS. It has been suggested that EPS inhibits the initial attachment of bacterial cells to the surface as well as to bacterial cells by weakening cell surface modifications or by reducing cell to cell surface interactions, though may not playing a direct role in the inhibition (Kim et al., 2009). It has been suggested that the polysaccharides present in the EPS modify abiotic as well as bacterial cell surfaces and mediate antibiofilm activity (Rendueles, Kaplan, & Ghigo, 2013). Polysaccharides also act as lectin inhibitors impacting lectine-dependent adhesion of bacteria and biofilm formation (Bernal & Llamas, 2012). Some polysaccharides are known to down regulate the expression of bacterial genes involved in biofilm formation (Kim et al., 2009). This is the first report on the antibiofilm activity of EPS from *Oceanobacillus*.

3.6.2. Evaluation of rheological properties

The concomitant decrease of viscosity of the EPS with shear rate revealed its pseudoplastic fluid behaviour (Fig. 4A). This pseudoplasticity was more profound up to a shear rate of 200–270 s^{-1}; thereafter, Newtonian behaviour was observed. The high viscosity of EPS is attributed to its polymeric structure and high molecular mass (Freitas et al., 2009b; Kanmani et al., 2011). The industrial process often involves a wide range of temperature and pH. Therefore, the EPS solution was subjected to varying temperatures (10–60 °C) at two different pH levels (3 and 7). At pH 3.0, a highly acidic condition, the glycosidic linkages may get hydrolysed resulting in lowering the viscosity of EPS. The EPS showed shear thinning behaviour at both low and neutral pH, making it a promising additive for the food industry, as it may provide suspension and sensory qualities in food products. Shear thinning is mainly caused by

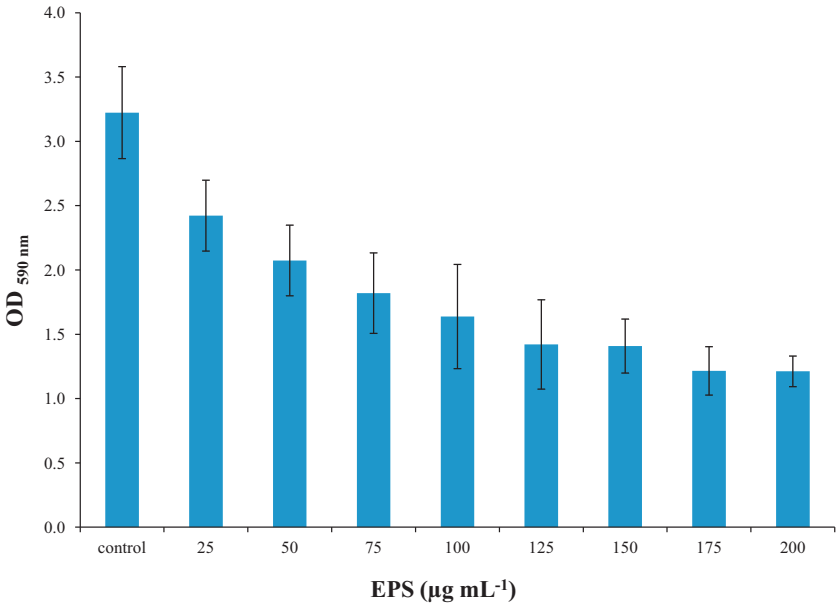

Fig. 3. Antibiofilm activity of EPS produced by *O. iheyensis*. Control represents biofilm of *S. aureus* without EPS.

Table 1
The effect of EPS produced by *O. iheyensis* on surface tension.

S. No.	Sample (filtered with 0.2 μm) (100 mL)	Amount of EPS (mg)	Surface tension (mN m^{-1})
1.	Zobell marine broth	0.0	51.53 ± 0.04
2.	12 h culture supernatant	5.7	50.17 ± 0.13
3.	24 h culture supernatant	19.9	49.95 ± 0.02
4.	48 h culture supernatant	39.6	40.80 ± 0.03

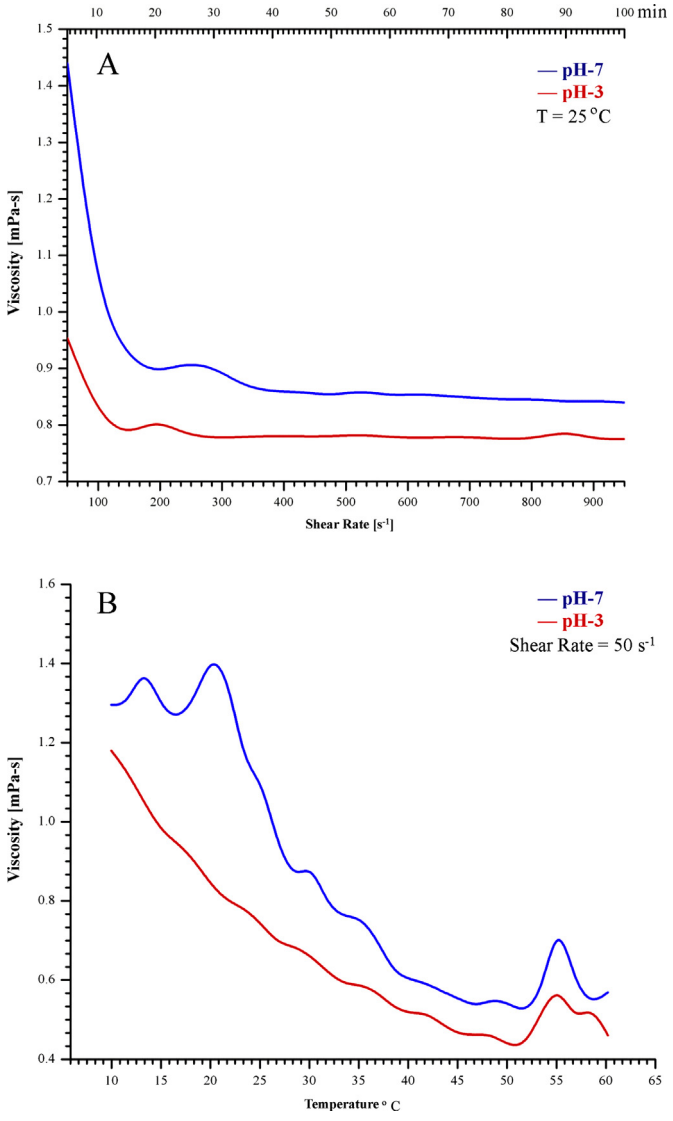

Fig. 4. Rheology of EPS produced by *O. iheyensis*. Effect of shear rate (at constant temperature 25 °C) (A) and temperature (at constant shear rate 50 s^{-1}) (B) on viscosity at different pH (3 and 7).

the breakdown of structural units in EPS by hydrodynamic forces generated during shear. Similarly, viscosity decreased with increasing temperature at both pH values (Fig. 4B). A similar pattern in the case of EPS from V. alginolyticus is reported (Muralidharan & Jayachandran, 2003). The low viscosity at higher temperature may be because of a decreased interaction between the molecules, which leads to a looser polymer structure (Freitas et al., 2009b). It has also been conjectured that the decrease in viscosity may be because, at higher temperatures, the different intermolecular arrangement causes modifications in the EPS tertiary structure Kanmani et al. (2011).

3.6.3. Emulsifying activity

A stable emulsion of the EPS was observed with hexadecane. This yielded 66.47% emulsifying activity with hexadecane after 30 min incubation. The emulsifying activity of EPS was measured before 30 min because emulsification will break within 30 min, due to the stability of the sample (Royan, Parulekar, & Mavinkurve, 1999). After 30 min the emulsifying activity was comparable to that of Vibrio campbellii (64.28%) but lower than V. fortis (71.64%) (Kavita et al., 2013). The formation of a clumpy solution in some of the commercial emulsifiers, in particular those with fatty acid components, limits their applications (Gutiérrez, Leo, Walker, & Green, 2009). In contrast, the EPS of *O. iheyensis* yields a clear solution in water, which indicates its potential application as a bioemulsifier. The presence of both hydrophilic (hydroxyl) and hydrophobic (aliphatic CH_2) functional groups may be responsible for its emulsifying property.

3.6.4. Biosurfactant properties

The filtered culture supernatant of *O. iheyensis* showed a reduction in surface tension compared to the medium, and the highest reduction was observed at 48 h (Table 1). We have also observed that the maximum production of EPS was at 48 h. It suggests that the reduction in surface tension is due to released EPS in cell free supernatant. It has been reported earlier that the reduction in surface tension in a marine bacterium, Planococcus maitriensis is correlated in the production of EPS (Kumar, Mody, & Jha, 2007). The EPS of *O. iheyensis* exhibited 20% lower surface tension than that of broth alone (Table 1). In a recent study, the culture supernatant of different marine bacterial isolates gave 7–16% reduction in surface tension (Bozo-Hurtado, Rocha, Malavé, & Suárez, 2012). In comparison, *O. iheyensis* shows a better efficiency in lowering the surface tension. Reduction in surface tension is the basic principle of detergents to be used for cleaning purposes. Thus, this EPS may have potential use as a biosurfactant. Generally, biosurfactants also have emulsifying property but all bioemulsifiers do not necessarily reduce surface tension (Karanth, Deo, & Veenanadig, 1999). However, the EPS of *O. iheyensis* shows properties of both biosurfactant and bioemulsifier.

4. Conclusion

The EPS produced by the marine bacterium *O. iheyensis* strain BK6 consists of three monosaccharides (mannose, glucose and arabinose). It comprises methyl groups, primary amines, halide groups, uronic acid and saccharides, and is thermostable. It exhibited characteristic TGA and DSC profiles. The antibiofilm activity of the EPS against the pathogenic strain of *S. aureus* revealed its potential pharmaceutical application. In addition, its pseudoplastic rheology, stable emulsifying activity and reduction in surface tension make it potentially important for industry. This is the first report giving a detailed characterisation and potential application of EPS from the genus *Oceanobacillus*.

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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.2013.08.099.

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