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Characterization of extracellular polymeric substances produced by micro-algae *Dunaliella salina*

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ABSTRACT

Extracellular polymeric substances comprised of average molecule size 1264.354 μm, exhibited characteristic diffraction peaks at 6.025°, 9.675°, 22.775° and 28.475° with *d*-spacing 14.74755, 9.36297, 3.88747 and 3.11512 Å, respectively. EDX confirms the presence of sulphate (2.7%) and ^{1}H NMR reveals uronic acid, primary amine, aromatic-compounds, halides, aliphatic alkyl and sulfides. EPSs were thermostable upto 270 °C with CI_{xrd} 0.12 and CI_{DSC} 0.18. The dynamic viscosity is significantly high at pH 3.0 and decreases concomitantly with shear rate, confirming pseudoplastic rheological property. MALDI TOF–TOF represents a series of masses in linear mode corresponding to mass of pentose and hexose with ions. The positive ion reflector mode exhibited low mass peaks (m/z) corresponding to oligosaccharide and higher peaks for polysaccharide consist of different ratio of pentose and hexose associated with ions. EPSs allow further exploration of *D. salina* as potential EPSs producer and make it a promising candidate for biotechnological and industrial exploitation.

1. Introduction

Extracellular polymeric substances (EPSs) are metabolic products, accumulating on the microbial cell surface, providing protection to the cells by stabilizing membrane structure against the harsh external environment and also serve as carbon and energy reserves during starvation. EPSs, a heterogeneous matrix of polymers composed of polysaccharides, proteins, nucleic acids and (phospho) lipids (McSwain, Irvine, Hausner, & Wilderer, 2005), are renewable resources representing an important class of biotechnological importance. EPSs are observed in bacteria (Freitas et al., 2009), cyanobacteria (Chi, Su, & Lu, 2007; Parikh & Madamwar, 2006) and marine microorganisms (Satpute, Banat, Dhakephalkar, Banpurkar, & Chopade, 2010). Exopolymers have also been reported from micro-algae *Dunaliella salina* (Mishra & Jha, 2009) and medicinal mushroom *Phellinus linteus* (Zou, Sun, & Guo, 2006). The polysaccharides of biological response modifiers can be isolated from bacteria, fungi, brown algae and photosynthetic plants (Leung, Liu, Koon, & Fung, 2006).

Microbial exopolymers have multifarious industrial applications (Kumar, Mody, & Jha, 2007). These exopolymers are used in food industries as thickeners and gelling agents to improve food quality and texture. In pharmaceutical industry, exopolymers can be used as hydrophilic matrix for controlled release of drugs, development of bacterial vaccines and to enhance nonspecific immunity. Improvement of water holding capacity of soil, detoxification of heavy metals and radio nuclides contaminated water and removal of solid matter from water reservoirs are proposed uses for cyanobacterial EPSs (Bender & Phillips, 2004). In recent years, interest in the exploitation of valuable EPSs has been increasing for various industrial applications and the attention towards exopolymer producing bacteria and cyanobacteria has greatly increased. EPSs are regarded as an abundant source of structurally diverse polysaccharides, some of which may possess unique properties for special applications like sludge settling and dewatering (Subramanian, Yan, Tyagi, & Surampalli, 2010). The increased demand of natural polymers for various industrial applications in past few years has led to sway interest in EPSs production by new sources and marine microbes (micro-algae and marine microorganism), already used as a source of products of high aggregated value such as pigments, osmoprotectant, metabolites, fatty acids and proteins, may also be exploited for the EPSs as biosurfactants and/or bioemulsifiers (Mishra & Jha, 2009; Satpute et al., 2010).

Micro-algae *D. salina* is the only eukaryotic photosynthetic organism can grow in the wide range of hypersaline environment (Mishra, Mandoli, & Jha, 2008). The ability of cells to survive and flourish in saline environment under the influence of osmotic stress has received considerable attention (Chen & Jiang, 2009) which make *D. salina* a perfect candidate for biotechnological exploration (Tafreshi & Shariati, 2009), molecular farming (Barzegari et al.,

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in press) and novel industrial applications (Francavilla, Trotta, & Luque, 2010). Micro-algal (*D. salina*) extracellular polymeric substances are a complex mixture of macromolecular polyelectrolytes containing primary amine-group, halide-group, aliphatic alkyl-group, aromatic compound, alkyl amine and/or cyclic amine with polysaccharides (Mishra & Jha, 2009).

The present study focuses on the analytical and rheological characterization of extracellular polymeric substances, produced by micro-algae *D. salina*. Despite of β-carotene, glycerol and other metabolites, EPSs make *Dunaliella* more promising candidate to play an important role in its biotechnological and industrial application as the resource of biosurfactants and/or bioemulsifiers. Emulsifiers from renewable resources have attracted attention and the emulsifying activity of *Dunaliella* exopolymer was determined by its strength in retaining the emulsion. The emulsifying activity of EPSs was 85.76% retention (Mishra & Jha, 2009), which is comparatively stable compared to that of other EPSs, produced by *Vibrio harveyi* (40% retention; Bramhachari & Dubey, 2006), *Halomonas* (25–60% retention) and commercially available surfactants viz. Tween 20 (65%), Tween 80 (60%) and Triton X-100 (65%) in tetradecane (Mata et al., 2006). The use of biosurfactants and bioemulsifiers represent a better alternative to overcome the toxicity of synthetic compounds as these are less toxic and mostly biodegradable (Satpute et al., 2010). The stable emulsifying activity makes *Dunaliella* exopolymer as an alternative of the commercially available chemical surfactants/emulsifiers.

2. Material and methods

Extracellular polymeric substances (EPSs) were isolated and purified from axenic culture of *D. salina* (Mishra et al., 2008). The emulsification activity, major functional groups analysis using FT-IR and monosaccharide composition were studied previously (Mishra & Jha, 2009).

2.1. Energy dispersive X-ray spectroscopy and particle size distribution

Elemental analysis of EPSs was done using energy dispersive X-ray spectroscopy (EDS or EDX; Oxford Instruments, UK) and particle size distributions were measured by laser diffraction (Malvern Mastersizer 2000, Malvern Ltd., Worcestershire, UK).

2.2. X-ray diffraction (XRD) analysis

X-ray diffraction (XRD) was performed on X-ray powder diffractometer (Philips X'pert MPD, The Netherlands) using PW3123/00 curved Ni-filtered CuKα (λ = 1.54056 Å) radiation generated at 40 kV and 30 mA with a liquid nitrogen cooled solid-state germanium detector to study the nature of EPSs using slow scan in $2\theta = 2–80^{\circ}$. 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 focus and divergence slit was 100 mm. Dried EPSs sample was mounted on a quartz substrate and intensity peaks of diffracted X-rays were continuously recorded with scan step time 1 s at 25 °C while *d*-spacings appropriate to diffracted X-rays at that value of θ was calculated with Bragg's law (Eq. (1)).

$$d = \frac{\lambda}{2 \sin \theta} \tag{1}$$

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

Crystallinity index (CI_{xrd}) was calculated from the area under crystalline peaks normalized with corresponding to total scattering area i.e. ratio of areas of peaks of crystalline phases to the sum of areas of crystalline peaks and amorphous profile (Eq. (2), Ricou, Pinel, & Juhasz, 2005).

$$CI_{xrd} = \frac{\sum A_{crystal}}{\sum A_{crystal} + \sum A_{amorphus}} \tag{2}$$

2.3. Thermal gravimetric analysis (TGA) and differential scanning calorimetric (DSC) analysis

TG and DSC analysis of EPSs were carried on Mettler Toledo TGA/SDTA System (Greifensee, Switzerland). Thermograms for TGA and DSC were obtained in the range of 30–400 and 25–600 °C respectively, under nitrogen atmosphere at rise of 10 °C min^{-1}. TG and DSC analysis were carried out by gradually raising the temperature, plotting weight (percentage) and heat flow against temperature respectively. The activation energy (E_a) was measured from Arrhenius equation (Eq. (3)) while enthalpy of transition (ΔH) and crystallinity of the exopolymers (CI_{DSC}) were calculated by following Eqs. (4) and (5) (Khanna & Kuhn, 1997) respectively.

$$E_a = -RT \ln \left(\frac{k}{A}\right) \tag{3}$$

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

$$\Delta H_{total} = KA \tag{4}$$

where ΔH is the enthalpy of transition, K is the calorimetric constant and A is area under the curve.

$$CI_{DSC} = \frac{\Delta H_{Net}}{\Delta H_{total}} \tag{5}$$

where ΔH_{Net} is difference between heat of crystallization and melting.

2.4. Rheological property analysis

Dynamic rheological measurement of EPSs solution (0.4% w/v in milliQ) was carried out on a rheometer (RS1, Haake Instruments, Karlsruhe, Germany) at different temperature, applied shear rate and pH with 1 ml sample volume. Measurements were carried out immediately after placing the sample on plate and to avoid loss due to evaporation at higher temperature, the outer surface of the samples were covered with silicon oil. All experiments were carried out in triplicates and slippage of gel due to applied stress was carefully avoided by selecting appropriate operation parameters.

2.5. Nuclear magnetic resonance (NMR) and scanning electron microscope (SEM)

The ^{1}H NMR spectra of EPSs were obtained in D_2O with Bruker Avance II 500 (Switzerland) operating at 500 MHz with net spinning 5000 rpm. Spectra were measured in the ppm range of 0–10. The morphology of exopolymer has been observed under a scanning electron microscope (SEM, LEO series VP1430, Germany) with an accelerated voltage of 10–20 kV.

2.6. MALDI TOF–TOF mass spectroscopy

The EPSs were dissolved in acetonitrile (5% w/v; 1 mg ml^{-1}), desalted and mixed with equal volume of matrix α-Cyano-4-hydroxycinnamic acid (10 mg ml^{-1}). MALDI TOF–TOF analysis was performed on an Applied Biosystem 4800 MALDI TOF–TOF analyzer with an Nd-YAG (neodymium-doped yttrium aluminium garnet) laser (355 nm, 200 Hz) operated in accelerated voltage (20 kV). Each spectrum was collected in two different modes, linear mode

Table 1
Elemental EDX microanalysis of EPSs obtained from *D. salina*. Data are expressed as both weight and atomic percents.

Element	Standard used	Weight%	Atomic%
C	$CaCO_3$	27.13	33.18
N	Not define	15.94	16.72
O	SiO_2	51.19	47.00
Na	Albite	2.07	1.32
Mg	MgO	0.64	0.38
P	GaP	0.34	0.16
S	FeS_2	2.70	1.24
Total		100.00	

Number of iterations = 6.

(150 cm) and reflector mode (300 cm) as an average of 1400 laser shots per spectrum. Reproducibility of the spectrum was checked from 6 spot-sets in each mode and the spectra were analyzed after centroid and de-isotoping using Data explorer software (Applied Biosystem, USA).

3. Results and discussion

3.1. Energy dispersive X-ray spectroscopy and particle size distribution

Extracellular polymeric substances constituted of particle sizes ranging from 49.492 ($d_{0.1}$) to 1634.192 ($d_{0.9}$) μm with an average size of 1264.354 μm ($d_{0.5}$) and 0.0651344 $m^2\,g^{-1}$ specific surface areas (Fig. S1). Energy dispersive X-ray spectroscopy (EDS or EDX) is an analytical technique used for the elemental analysis of a sample and it is one of the variants of X-ray fluorescence spectroscopy. EDX relies on the investigation of a sample through interactions between electromagnetic radiation and matter, analyzing X-rays emitted by the matter in response to be hitted with charged particles (Goldstein et al., 2003). Elemental quantitative analysis done by EDX revealed the weight and atomic percentage of elements present in EPSs (Table 1 and Fig. S2). EDX analysis confirms the presence of sulphate residue (2.7% w/w), a characteristic of exopolysaccharides produced by eukaryotic algae (Gudin & Thepenier, 1986).

3.2. X-ray diffraction (XRD) analysis

X-ray powder diffraction (XRD) is a rapid analytical technique most widely used for phase identification of a crystalline material. A polymer can be considered partly crystalline and partly amorphous. The XRD profile of EPSs obtained from *D. salina* (Figs. 1 and S3) exhibited the characteristic diffraction peaks at 6.025°, 9.675°, 22.775° and 28.475° with interplanar spacing (*d*-spacing) 14.74755, 9.36297, 3.88747 and 3.11512 Å, respectively. It is first XRD analysis of the EPSs so far and PXRD pattern predict that EPS is amorphous in nature with 0.12 crystallinity index. Crystalline parts give sharp narrow diffraction peaks while amorphous component gives a broad peak. It is difficult to interpret broad amorphous peaks of several amorphous polymer in X-ray scattering profile (Shimazu, Miyazaki, & Ikeda, 2000) and hence the ratio between these intensities is used to calculate the amount of crystallinity in the material. EPSs, obtained from *D. salina*, are found amorphous in nature with CI_{xrd} 0.12. The 12% crystalline domains act as a reinforcing grid and improve the performance over a wide range of temperature as observed in TG and DSC analysis.

3.3. TG and DSC analysis

The applicability of polysaccharides is largely dependent on its thermal and rheological behavior. Thermogravimetric analysis is a simple analytical technique that measures the weight loss of a material as a function of temperature. TGA showed that degradation of EPSs obtained from *D. salina* takes place in two well-differentiated steps, however EPSs of cyanobacterial origin showed the degradation in three steps (Parikh & Madamwar, 2006). Fifteen percent of total EPSs weight loss from 30 to 124 °C was recorded for phase 1 degradation, thereafter second phase of degradation (54.6%) was observed with maximum loss at ≥240 °C (Fig. 2(a)) temperature. Phase 1 degradation may be due to water evaporation during heating process while second phase of degradation is attributable to thermal decomposition same as other study (Parikh & Madamwar, 2006).

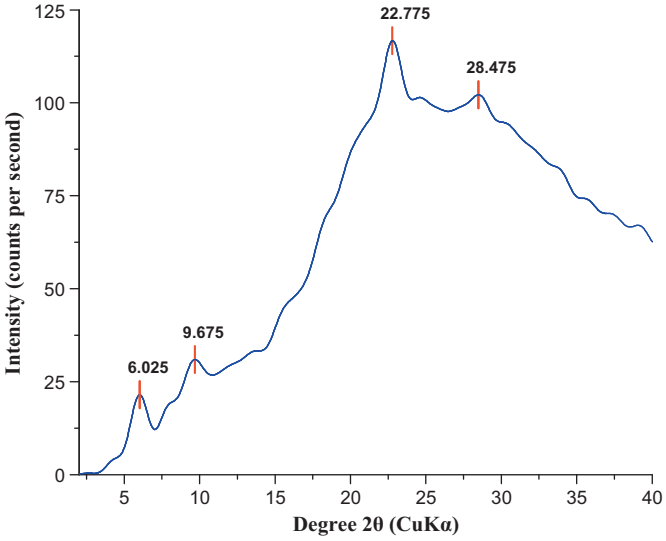

Fig. 1. Representative PXRD profile of EPSs isolated from *Dunaliella salina*.

As temperature increases, an amorphous solid will become less viscous and at a particular temperature the molecules obtain enough freedom of motion to spontaneously arrange themselves into a crystalline state, known as the crystallization temperature (Dean, 1995). This transition from amorphous solid to crystalline solid is an exothermic process and differential scanning calorimetric analysis showed a significant thermal transition of EPSs (Fig. 2(b)). DSC thermogram showed characteristic exothermic transition of exopolymer with crystallization temperature (T_c) 95.88 °C (onset temperature 59.56 °C) and 475.46 mJ latent energy for crystallization followed by an endothermic transition of melting. The melting temperature (T_m) of EPSs was found 270.45 °C (onset temperature 253.52 °C) with 193.06 mJ latent energy for melting. The activation energy (for *n*th order reaction) of exothermic transition was 60.79 ± 0.35 kJ mol^{-1} while for endothermic transition, it was found quite higher 292.62 ± 1.56 kJ mol^{-1}. The activation energy of EPS isolated from marine cyanobacterium *Cyanothece* sp. was found to be 149.6 kcal mol^{-1} (Shah, Ray, Garg, & Madamwar, 2000) while 450–490 kJ mol^{-1} for *Oscillatoria* sp. and *Nostoc carneum* (Parikh & Madamwar, 2006). EPSs exhibited cross linking characteristics at high temperature ranging 385–500 °C, thereafter its gets oxidized. The thermostability of EPSs was observed up to high temperature (270 °C). In contrast with PXRD, slightly higher crystalline index (CI_{DSC} 0.18) was observed for EPSs from DSC thermogram and it may be because of uncertainties in placing baseline for area integration (Khanna & Kuhn, 1997).

3.4. Rheological property analysis

The dynamic viscosity (η) of EPSs decreased concomitantly with shear rate (γ) at pH 3.0, showing pseudoplastic rheological property

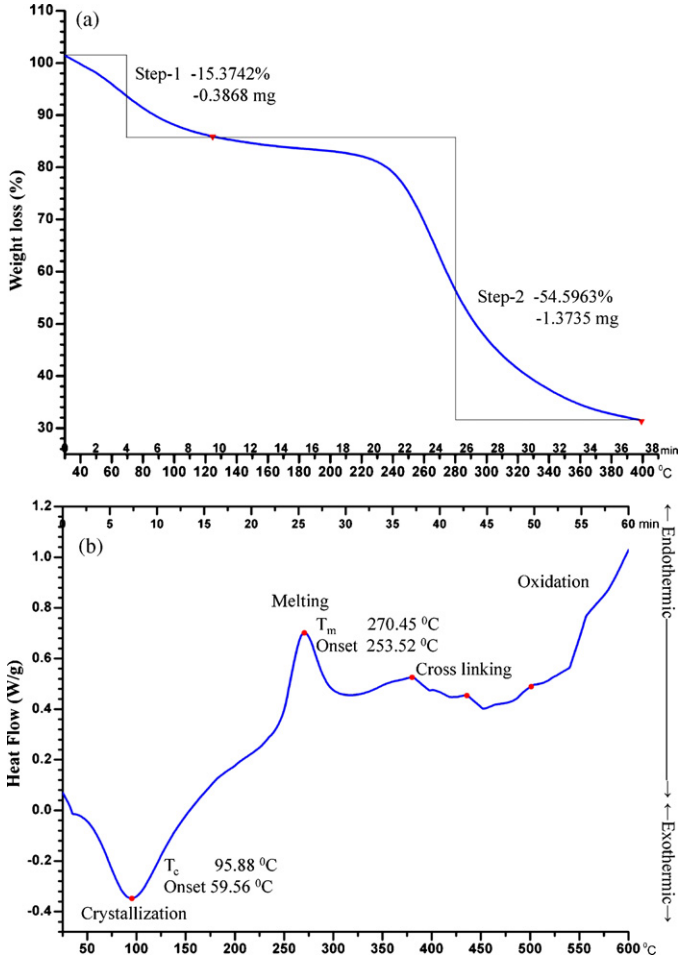

Fig. 2. Thermogravimetric (a) and DSC (b) thermogram of EPSs obtained from *Dunaliella salina* at heating rate of 10 °C.

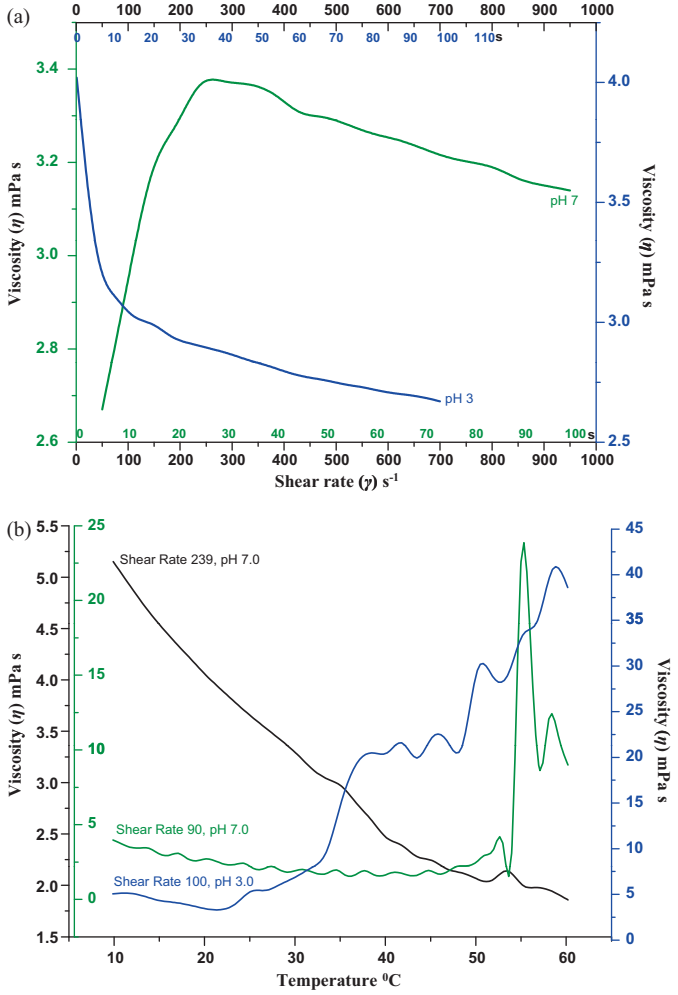

Fig. 3. Effect of (a) shear rate and (b) temperature on viscosity of EPSs isolated from *Dunaliella salina*.

while at pH 7.0, viscosity increases and reached maxima at 239 (s^{-1}) shear rate, thereafter decline gradually (Fig. 3(a)). In contrast to pH 3.0 (700 shear rate s^{-1}), EPSs showed significant viscosity limit up to 950 (s^{-1}) shear rate at pH 7.0. Viscosity of EPSs is significantly high at low pH (3.0) compare to neutral pH (7.0), independent to shear rate (i.e. 0–50 s^{-1}) may be due to its uronic acid content (Béjar, Llamas, Calvo, & Quesada, 1998) as observed in ^{1}H NMR and showed pseudoplastic characteristic like halophilic bacterial EPSs, isolated from *Idiomarina fontislapidosi*, *I. ramblicola* and *Alteromonas hispanica* (Mata et al., 2008). The pseudoplastic characteristic of *Dunaliella* EPSs at pH 3.0 is because it has been reported that there is an increase in viscosity at low cation concentration (Parker, Schram, Plude, & Moore, 1996). Low ion concentration reflects the change in polymer configuration and initial stage of chain aggregation with large amount of trapped solvent. After maximum compatible aggregation in solution is attained, a rearrangement of aggregates in more condensed structures (precipitates) is likely to occur (Parikh & Madamwar, 2006; Parker et al., 1996).

Viscosity of EPSs decreased concurrently with temperature at pH 7.0 and constant shear rate 239 (s^{-1}), however at 52–54 °C, a sudden increases in viscosity was observed (Fig. 3(b)). EPSs showed low and steady viscosity with low shear rate (90 s^{-1}) at pH 7.0 up to 54 °C, a sudden increase in viscosity was found at 55 °C leading to decline thereafter. An increase in viscosity was recorded for EPSs at pH 3.0 (shear rate 100 s^{-1}) with temperature up to 56 °C, after that declines on further increase of temperature. The viscosity of the EPS extracted from *Vibrio alginolyticus*, decreased throughout a range of increasing shear rate, and with an increase in temperature the viscosity also dropped markedly (Muralidharan & Jayachandran, 2003). They also demonstrated that EPS was unstable at high temperatures and high pH. Other than bacterial EPSs, dynamic viscosity decreased with increasing shear rate for exopolysaccharide (agar) isolated from seaweeds *Gracilaria acerosa* (Prasad et al., 2007). It was observed that EPSs obtained from halophilic species were low in viscosity and pseudoplastic in the behavior (Mata et al., 2008).

3.5. NMR and SEM

The ^{1}H NMR spectra of EPSs, obtained from *Dunaliella* culture, reveal characteristic chemical shifts (ppm) and corresponding functional groups (Fig. 4). The ppm 5.1–5.4 and 4.8–4.9 are attributed to α- and β-anomeric carbon of hexose or pentose, respectively. A spectrum at 4.0 ppm is assigned to hydrogen next to the functional –OH group while uronic acid is observed at 2.3 ppm. A stretching of N–H group is observed at 1.3 ppm while alkyl halide group at 3.1 ppm. Spectra at 2.0 and 2.7 ppm correspond to the functional group acetyl amine of hexose or pentose sugar moiety. Apart from polysaccharide functional groups, others functional groups related to alkene, alkyne, aliphatic, aromatic compound, CH–O, CH–N and S–H were also observed (Fig. S4). The NMR spectrum of EPSs confirms the presence of polysaccharides, uronic acid, primary amine group, aromatic compound, halide group, aliphatic alkyl group and sulfides. The FT-IR-spectra confirmed the presence of primary amine-group, aromatic compound, halide-group,

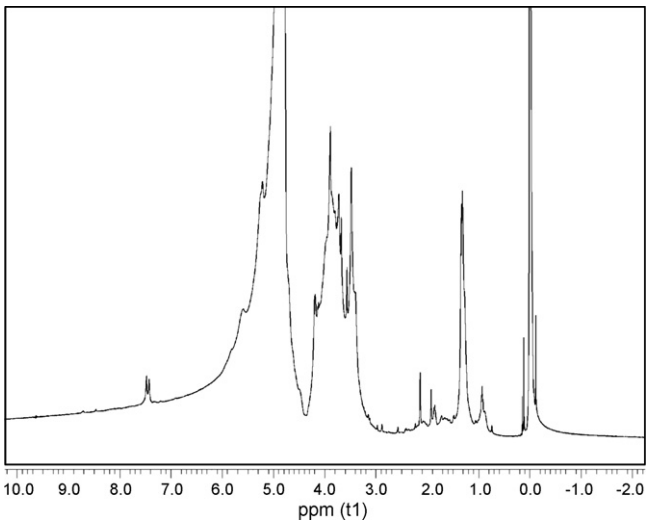

Fig. 4. Proton nuclear magnetic resonance (^{1}H NMR) spectra of EPSs extracted from *Dunaliella salina*.

aliphatic alkyl-group and polysaccharides, as a resultant presence of alkyl amine and/or cyclic amine with polysaccharides in EPSs of *D. salina* was depicted (Mishra & Jha, 2009). The presence of acetyl groups renders the EPSs somewhat hydrophobic, which might also contribute to their emulsifying capacity (Mata et al., 2006) as observed previously (Mishra & Jha, 2009). The presence of uronic acids and sulfates confers an overall negative charge and acidic property to the polymer (Jain, Raghukumar, Tharanathan, & Bhosle, 2005). Acidic polysaccharides are common in marine organisms, also reported from bacteria, cyanobacteria, and diatoms (Khandeparkar & Bhosle, 2001; Philippis & Vincenzini, 1998). Negatively charged cyanobacterial EPS containing uronic acids exhibited a high metal-complexing capacity (Philippis, Margheri, Materassi, & Vincenzini, 1998). The presence of uronic acids and sulfates confers an overall negative charge and acidic property to the exopolymer and sulfated EPSs are of biotechnological importance. It has been clear from SEM images (Fig. S5) that exopolymer is compact with small pore size distribution. Compactness with porosity was also observed in the EPS extracted from *Azotobacter* sp. (Gauri, Mandal, Mondal, Dey, & Pati, 2009).

3.6. MALDI TOF–TOF analysis

Matrix assisted laser desorption–ionization mass spectroscopy is a convenient method for rapid and sensitive structural analysis of oligosaccharides (Harvey, 1999). The MALDI TOF–TOF mass spectroscopy was optimized for EPSs and fragmentation peaks are detected for positive ion mode only. No peaks were observed for negative ion linear or reflector mode. Linear mode was found suitable for the oligosaccharide while positive ion reflector mode for the polysaccharide analysis. MALDI TOF–TOF mass spectroscopy of EPSs (Fig. S6) represents a series of masses m/z 177.8892, 227.7699 and 274.1352 in linear low range mode which correspond to de-protonated hexose sugar and hexose sugar with matrix (size 47.2878 as observed in reflector mode). Besides this, masses m/z 300.2427 and 384.0726 are also observed in positive ion linear mid range mode, corresponding to mass of two pentose and two hexose (with Mg ion) respectively. The positive ion reflector mode exhibited low mass peak m/z 361.7294 and 662.2907 corresponding to two hexose and a tetra-saccharide (2 hexose and 2 pentose) respectively and higher mass peaks at m/z 1101.222, 1651.635, 1939.387 and 2272.089, corresponding to polysaccharides consisting of different ratio of pentose and hexsose sugar associated with ions like Mg, S, Na, P, etc. present in EPSs, as detected by EDX. It is the first MALDI TOF–TOF analysis of EPSs so far, whereas MALDI TOF mass spectroscopy for the bacterial EPS was reported recently (Gauri et al., 2009).

The presence of pentose sugar, which is usually absent in polysaccharides of prokaryotic origin and quite unique among cyanobacteria (Parikh & Madamwar, 2006), is remarkable. Previously, we have detected four constituent monosaccharides viz. galactose, glucose, xylose and fructose in different combinations and ratio by HPLC analysis, after complete acidic hydrolysis of EPSs (Mishra & Jha, 2009). Monosaccharides detected in EPSs of *Dunaliella* can be categorized as aldohexoses (glucose and galactose), ketohexose (fructose) and pentose (xylose) sugars. Though mass spectroscopy cannot distinguish diastereomers but it indicates the number of sugar moiety in an oligomer.

4. Conclusion

Extracellular polymeric substances of *D. salina* can play an important role in its biotechnological and industrial application. In this study, EPSs are characterized by advanced analytical methods and its rheological property is also studied. XRD profiling and MALDI TOF–TOF analysis for EPSs is the first report so far. XRD profile and interplanar spacing (*d*-spacing) is the basic characteristic of a polymer useful to compare or study the nature of EPSs isolated from different sources in future. The EPSs are found thermo stable up to high temperature (270 °C) which enables it for additional characteristic for further applications. The exopolymer is exceptionally attractive for industrial application because of high viscosity of its solution at acidic pH. The presence of uronic acids and sulfates confers an overall negative charge and acidic property to the exopolymer and sulfated EPSs are also of biotechnological importance. EPSs may allow further exploration of *D. salina* and make it a promising candidate for commercial exploitation. Studies on the biotechnological importance and ecological significance of the extracellular polymeric substances of *D. salina* deserve further attention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.08.067.

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