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Isolation and structural characterization of biosurfactant produced by an alkaliphilic bacterium *Cronobacter sakazakii* isolated from oil contaminated wastewater

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ABSTRACT

Production of biosurfactant from an alkaliphilic bacterium *Cronobacter sakazakii* (accession no. JN398668) was screened by haemolytic assay, emulsifying activity and surface tension measurement. Biosurfactant, comprised of total sugars (73.3%), reducing sugars (1.464%), protein (11.9%), uronic acid (15.98%) and sulfate (6.015%), showed low viscosity with pseudoplastic rheological behavior and exhibited significant emulsification activity with oils and hydrocarbons. A series of low and mid range mass peaks (m/z) corresponding to mono-, di-, tri- and oligosaccharides were detected in the positive ion reflector mode of MALDI TOF–TOF MS. GC–MS analysis revealed composition of monosaccharide moieties (w/w) *viz.* glucose (14%), mannose (24%), galactose (14%), xylose (20%) and arabinose (1.9%). ^{1}H NMR, FT-IR and EDX analyses confirmed the characteristic various functional groups, bonds and elements respectively. Thermostability (up to 260 °C) and CI (0.456) were determined by TG and DSC analyses. Inherent properties of biosurfactant make it a potential candidate for bioremediation of oil and hydrocarbons.

1. Introduction

Microbially derived biosurfactants are heterogenous and structurally diverse group of surface active molecules produced by a wide variety of bacteria, yeast, phytoplankton, algae, cyanobacteria and filamentous fungi from different environmental habitats which either adhere to cell surface or excreted extracellularly (Chi, Su, & Lu, 2007; Kumar, Mody, & Jha, 2007; Makker & Cameotra, 1998; Mandal, Singh, & Patel, 2011; Mishra & Jha, 2009; Parikh & Madamwar, 2006; Rosenberg & Ron, 1999; Sahoo, Datta, Biswas, & Choudhury, 2010; Singh et al., 2011). Biosurfactants are amphiphilic compounds (with polar and non-polar moieties) comprised of glycolipids, phospholipids, lipopeptides and polymeric compounds (Banat, Makkar, & Cameotra, 2000), tend to separate at interfaces and reduced the surface tension (Bramhachari et al., 2007; Desai & Banat, 1997).

In recent years, interest in the exploitation of valuable biosurfactants has been increasing for various industrial applications and the attention towards biosurfactant producing extremophilic bacteria has greatly enhanced. Extreme environments are bioresources of potential microorganisms that secrete new bioactive compounds and biosurfactants (Rodrigues, Teixeira, Van der Mei, & Oliveira, 2006). Extremophiles are of interest because of their potential for environmental applications, especially as source of emulsifiers for bioremediation of hydrocarbon and toxic metal from contaminated soils and wastewater in extreme conditions (Nicolaus, Kambourova, & Oner, 2010). Biosurfactants have different chemical structures, compositions and a wide range of biotechnological applications in dairy, food, beverage, cosmetics, detergent, textile, paint, mining, petroleum, paper pulp and pharmaceutical industries (Bhaskar & Bhosle, 2005; Cameotra & Makker, 2004; Rodrigues et al., 2006; Singh & Cameotra, 2004). Environmental imbalance, created by crude oils, hydrocarbons and toxic metals can be remediated by biosurfactant effectively as it forms stable emulsion, adsorbent or amalgam (Calvo, Manzanera, Silva-Castro, Uad, & Gonzalez-Lopez, 2009; Kumar, Mody, & Jha, 2007). Biosurfactants are ecologically accepted, low toxic, biodegradable and effective in a wide range of extreme conditions including temperature, pH and salinity (Banat, Makkar, & Cameotra, 2000; Bramhachari et al., 2007; Hazra et al., 2011; Rosenberg & Ron, 1999; Singh & Cameotra, 2004) compared to chemical surfactants.

There are few studies on biosurfactant production from extremophiles, however no reports on alkaliphiles so far. The present work involves analytical and rheological characterizations of biosurfactant from an alkaliphilic bacterium *Cronobacter sakazakii*, isolated from oil contaminated wastewaters. *C. sakazakii* is reported to be present as an opportunistic pathogen widely found in an environment especially in infant formulae as contaminant (Iversen et al., 2007). Two non-pathogenic strains of *C. sakazakii* were reported from plant roots by Schmid et al. (2009). Emulsifiers

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from renewable resources have attracted attention and the emulsifying activity of extracted biosurfactant was determined by its strength in retaining the emulsion. The stable emulsifying activity makes this biosurfactant as an alternative of the commercially available chemical surfactants/emulsifiers.

2. Materials and methods

2.1. Sample collection and isolation of alkaliphilic bacteria

Alkaline soil, alkaline effluent, alkaline sludge and oil industry wastewater samples were collected from Jhagadia, GIDC (21°39.22″N 73°8.15″E) and Gondal, GIDC (21°58.95″N 70°47.24″E) of Gujarat (India). Samples were inoculated into Horikoshi medium (HM), pH 10 for enrichment of bacteria. Enriched samples were 10-fold diluted, spread on the Horikoshi agar plates containing ($g\,l^{-1}$) glucose 10, peptone 5, yeast extract 5, K_2HPO_4 1, $MgSO_4{\cdot}7H_2O$ 0.2, Na_2CO_3 10 and agar 20, pH 10. Plates were incubated at 30 °C for 24 h and individual colonies were picked up and further streaked on Horikoshi agar plate in order to obtain pure culture. All the bacterial isolates were maintained on Horikoshi agar slant at 4 °C for subsequent experiments.

2.2. Screening for biosurfactant producer

2.2.1. Haemolytic assay

Haemolytic assay was performed for screening of biosurfactant producing bacteria (Plaza, Zjawiony, & Banat, 2006). For this, isolated alkaliphilic bacteria were streaked onto blood agar plates containing ($g\,l^{-1}$) trypticase 10, beef extract 3, NaCl 5 and agar 15. After autoclaving, 5% (v/v) sterile human blood was added (pH 10). Plates were incubated at 30 °C for 24 h and visually inspected for zone of clearance around the colony.

2.2.2. Production of biosurfactant

A loopful of alkaliphilic bacterium RJ-06 was transferred to Erlenmeyer's flask of 250 ml containing 100 ml HM broth of pH 10 and incubated for 24 h at 30 °C on a rotary shaker at 120 rpm for the preparation of seed culture. Biosurfactant was produced by inoculating 2% (v/v) seed culture (optical density of about 2.0 at 600 nm) in a 1000 ml Erlenmeyer's flask containing 500 ml SPY broth containing ($g\,l^{-1}$) sucrose 30, peptone 5 and yeast extract 1, pH 10 and incubated for 7 days at 30 °C on a rotary shaker at 120 rpm. Samples from production medium were removed at every 24 h interval and centrifuged (13,000 × *g*) at 4 °C and for 20 min in order to remove the bacterial cells. The obtained supernatant was acidified up to pH 2.0 using 6 M HCl, kept at 4 °C for 12 h and centrifuged (13,000 × *g*; 20 min, 4 °C). Alcohol (isopropanol) precipitation of the supernatant was carried out for the recovery of the biosurfactant. Dry weight of the crude product was measured. The crude product was dialysed for 2 days at 4 °C (12,000 Da cut off dialysis tubing, Sigma) and lyophilized.

2.2.3. Surface tension and rheological measurement

Samples of cell-free supernatant obtained at different time intervals were tested for their surface active properties. The surface tension of the obtained supernatant was measured by using du Nouy ring tensiometer (Data physics, Germany) at 30 °C (surface tension of distilled water and SPY medium was measured as control). Percentage reduction in surface tension was calculated by the following equation (Pansiripat et al., 2010):

$$\%\,\text{surface tension reduction} = \frac{(\gamma_m - \gamma_c)}{\gamma_m} \times 100$$

where γ_m is the surface tension of the SPY medium and γ_c is the surface tension of the centrifuged sample.

Viscosity measurement of the biosurfactant sample (0.5%, w/v) was carried out using a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany).

2.2.4. Measurement of emulsification index

For measuring emulsification index, hydrocarbons or oils were added to aqueous phase containing the biosurfactant (0.1%, w/v), in a ratio of 3:2, v/v, followed by vigorous agitation on a cyclo-mixer for 2 min. The oil, emulsion and aqueous layers were measured at every 24 h interval up to 240 h. The emulsification index was noted with respect to time (Cooper & Goldenberg, 1987) and is represented accordingly, *i.e.* the emulsification indices after 24 h, 48 h, 72 h, etc. were represented as E_{24}, E_{48}, E_{72} respectively.

$$E_{24}\,(\%) = \frac{h_{\text{emulsion}}}{h_{\text{total}}} \times 100$$

2.2.5. Chemical characterization of biosurfactant

Lyophilized biosurfactant was subjected to chemical analysis for estimation of total sugars (Morris, 1948), reducing sugars (Nelson, 1944), sulfate (Dodgson & Price, 1963), protein (Bradford, 1976), uronic acid (Knutson & Jeanes, 1968) and C, H, N and S analyses (Perkin Elemer, CHNS analyzer-2400, USA). The monosaccharide composition of biosurfactant was determined by analyzing derivatised samples using GC–MS (Siddhanta et al., 2001).

2.2.6. Molecular mass and MALDI TOF–TOF mass spectroscopy

The molecular weight of product was determined by gel permeation chromatography (7.8 mm i.d. × 300 mm stainless steel; GPC, Model Alliance 2695, Waters, USA) with guard column. About 50 μl of 0.1% product was loaded to GPC column ultrahydrogel 120 and ultrahydrogel 500 at 30 °C. Elution was monitored by a refractive index detector (2414). The column was calibrated with standard dextran (molecular weight, 5200–668,000 kDa) from PSS, USA. The biosurfactant was eluted with HPLC grade water and operated isocratically at a flow rate of 1.0 ml min^{-1}. Purified biosurfactant was prepared (5 mg ml^{-1}) in acetonitrile (50%, v/v) and mixed with equal volume of matrix α-Cyano-4-hydroxycinnamic acid (10 mg ml^{-1}). MALDI TOF–TOF analysis was carried out on MALDI TOF–TOF analyser (Applied Biosystem 4800, USA) with an Nd-YAG (neodymium-doped yttrium aluminium garnet) laser (355 nm, 200 Hz) operated in an accelerating voltage 20 kV. Each spectrum was collected in positive ion reflector mode with an average of 1400 laser shots per spectrum (Mishra, Kavita, & Jha, 2011). Reproducibility of the spectrum was checked from triplicate of six spot-sets and the spectra were analyzed after centroid and de-isotoping using Data explorer software (Applied Biosystem, USA).

2.2.7. FT-IR and ^{1}H NMR analyses

Fourier transform infrared spectroscopy (FT-IR) is used to elucidate the chemical structure of unknown samples by identifying type of functional groups. The sample for FT-IR analysis was prepared by grinding 1.0 mg of lyophilized biosurfactant with 100 mg of dry KBr followed by pressing the mixture with 7500 kg weight for 30 s to obtain translucent pellet. FT-IR spectrum was recorded in 4000–400 cm^{-1} region on a GX-FTIR system (Perkin Elmer, USA). ^{1}H NMR spectrum was recorded on a Bruker Avance-II spectrometer, Switzerland, at 500 MHz. Biosurfactant was dissolved in the D_2O (50 mg ml^{-1}) and spectra were recorded at 25 °C with 5000–5200 accumulations, pulse duration 5.9 μs, acquisition time 1.2 s and relaxation delay 6 μs.

2.2.8. Thermal gravimetric (TG) and differential scanning calorimetric (DSC) analyses

TG and DSC analyses of lyophilized biosurfactant were carried out with Mettler Toledo TGA/SDTA System (Greifensee, Switzerland). About 5–8 mg of lyophilized sample was loaded in a platinum pan and its energy level was scanned in the ranges of 30–480 °C and 30–450 °C respectively under a nitrogen atmosphere, with a temperature gradient of 10 °C min^{-1}. Both analyses were performed under gradual increase in temperature, plotting the weight percentage and heat flow against temperature respectively. The activation energy (E_a) was calculated using Arrhenius equation, $E_a = RT \ln(k/A)$, 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 coefficient.

2.2.9. Particle size distribution and energy dispersive X-ray spectroscopy (EDX)

Biosurfactant sample 0.5% (w/v) was taken for measurement of particle size distribution by laser diffraction (Malvern Mastersizer 2000, Malvern Ltd., Worcestershire, UK). Quantitative elemental analysis of biosurfactant was conducted using biosurfactant sample (5–7 mg) attached on aluminium stuff by SEM-EDX (SEM-EDX, Oxford Instruments, UK), which revealed the weight and atomic percentage of different elements present in the sample.

2.2.10. Identification of alkaliphilic bacterial strain RJ-06

Molecular identification was done using 16S rRNA gene sequence analysis. Genomic DNA was extracted by cethyl trimethyl ammonium bromide buffer [CTAB 2%, NaCl 1.4 mM, EDTA 50 mM, Tris 100 mM, PVP 20%] method. PCR amplification of partial 16S rRNA gene sequences was performed using the forward and reverse primers as 8f (fD1) 5′-AGA GTT TGA TCC TGG CTC AG-3′ and (rP2) 5′-ACG GCT ACC TTG TTA CGA CTT-3′ respectively. The reaction mixture of PCR amplification contained 1× PCR buffer, 200 μM dNTPs, 1.5 U Taq DNA polymerase, 20 pmol of each primers (Sigma, India) and 50 ng of genomic DNA. Amplification was performed in a thermal cycler (Bio-Rad MyCycler, California, USA) for an initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min and a final extension at 72 °C for 5 min. The purified PCR product was sequenced and the 16S rRNA gene sequence was compared with GenBank using the BLASTn program.

3. Results and discussion

3.1. Isolation, screening and quantitative determination of biosurfactant

A total of 43 isolates were obtained based on distinctly different colony morphology and all were screened for biosurfactant production. Only 6 isolates showed clear zones around bacterial colony on blood agar plates (S1). The cell-free culture supernatant of strain RJ-06 grown in liquid SPY medium showed the highest reduction of surface tension from 69.03 to 46.07 mN/m as compared to other isolates (S1). On the basis of these observations, strain RJ-06 was selected for further study. The bacterium RJ-06 showed maximum production of biosurfactant with a concentration of 3.15 g l^{-1} after 72 h.

3.2. Rheological measurements

The flow curve showed the pseudoplastic characteristics of solution of the biosurfactant (Fig. 1). The viscosity was found to be 0.429 Pa s^{-1} at 0.01 s^{-1} shear rate and 0.00429 Pa shear stress and which decreased concomitantly with an increase in the shear rate. Similarly, bioemulsifier of *Halomonas eurihalina* strain V2-7 exhibited pseudoplastic performance and viscosity decreased with increasing shear rate (Martinez-Checa, Toledo, Mabrouki, Quesada, & Calvo, 2007). The shear thinning behavior of the biosurfactant could be useful for biotechnological applications.

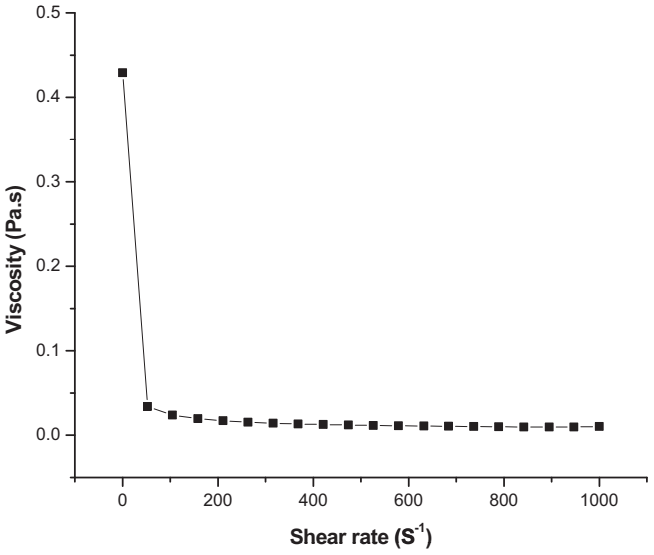

Fig. 1. Effect of shear rate on viscosity of biosurfactant (0.5%, w/v) produced by alkaliphilic bacterium RJ-06.

3.3. Emulsification activity of biosurfactant

The emulsifying activity is determined by its strength in retaining the emulsion of hydrocarbons or oils in water. The extracted biosurfactant efficiently emulsified with aliphatic, aromatic hydrocarbons and oils (Table 1). Formation of stable emulsion was observed with xylene, cyclohexane, cyclooctane, toluene, carbon tetrachloride, dichloromethane, cotton seed, jojoba and groundnut oil at 1 mg ml^{-1} biosurfactant concentration, which was reflected by the high emulsification indices in the range from 100% to 62% up to 240 h. It had a vital property of dissolving readily in water having a broad pH range of 2–12 and produced clear solution. Some commercial emulsifiers, particularly those with fatty acid components, tend to form clumpy solution that limits their applications (Gutierrez, Leo, Walker, & Green, 2009). The present study also revealed that emulsion indices and stability of emulsion were quite similar in alkaline, neutral and acidic conditions. The uronic acid and proteinaceous component of bacterial biosurfactant played an important role in the emulsification of hydrocarbons and oils, apart from functional groups

Table 1
Emulsification activity of biosurfactant (1 mg ml^{-1}) with hydrocarbons and oils.

Hydrocarbons and oils	Emulsifying index (E_{24})	Emulsifying index (E_{240})
Xylene	70	62
Cyclohexane	62	55
Cyclooctane	75	–
Toulene	80	62
Carbon tetrachloride	65	62
Dichloromethane	65	62
Kerosene	72	–
Cotton seed oil	90	62
Jojoba oil	100	62
Groundnut oil	90	62

(acetyl) present in the biopolymer, which also provide hydrophobicity, imparting enhanced emulsifying activity (Bramhachari et al., 2007).

3.4. Chemical characterization of biosurfactant

Total sugars, reducing sugars, uronic acid, protein and sulfate content were quantified as 73.3%, 1.464%, 15.98%, 11.9% and 6.015% respectively. Biosurfactant is heteropolysaccharide in nature, constituted of five monosaccharide moieties *viz.* (w/w) glucose (14%), galactose (14%), mannose (24%), xylose (20%) and arabinose (1.9%). The C, H, N and S analyses revealed presence of 42.27% carbon, 6.21% hydrogen, 7.26% nitrogen and 2.37% sulfur. Biosurfactants produced from *Marinobacter* sp., *Vibrio* sp., *Acetobacter* sp., *Halomonas* sp., *Bacillus* sp., *Pseudomonas* sp., *Corynebacterium* sp. and *Halobacter* sp. comprised of carbohydrate, uronic acid, protein and sulfate (Bramhachari et al., 2007; Calvo et al., 2009). Biosurfactant reported in the present study can be classified under polysaccharide–protein complex (polymeric microbial surfactant) (Karanth, Deo, & Veenanadig, 1999). The extracellular polysaccharide containing emulsifiers (emulsans) of two *Acinetobacter calcoaceticus* strains, RAG-1 and BD4, has been studied extensively by Kaplan, Rosenberg, Jann, and Jann (1985), which consisted of polysaccharides and proteins. Carboxyl group and sulfate group provide overall negative charge to the polymer, thereby imparting binding and adsorptive properties for divalent cation by electrostatic interactions (Bramhachari et al., 2007). Proteins present in biosurfactant play an important role in emulsification with hydrocarbons and oils (Bramhachari et al., 2007; Fazio, Uhlinger, Parker, & White, 1982; Sutherland, 2001). These chemical properties are accountable for bioremediation potential with respect to oils, toxic chemicals and hydrocarbons from contaminated soil and wastewaters.

3.5. Molecular mass and MALDI TOF–TOF mass spectroscopy

The GPC chromatogram generated a single peak corresponding to 3760 kDa approximately, with 1.017 polydispersity and retention time 12.39 min (S2). Biosurfactants are divided into low-molecular and high molecular weights, having different chemical structures and surface properties. High molecular weight biosurfactants, produced from *Bacillus* sp., *Acinetobacter* sp. and *Pseudomonas* sp., were used as emulsifiers and flocculants (Banat, Makkar, & Cameotra, 2000; Rosenberg & Ron, 1999). Matrix assisted laser desorption–ionization mass spectroscopy was used previously for the analysis of EPS (Mishra, Kumari, & Jha, 2011; Singh et al., 2011). MALDI TOF–TOF MS analysis of purified biosurfactant was done for the first time, so far and fragmentation peaks were observed for positive ion reflector mode, as this mode was found to be suitable for the oligosaccharide analysis (Mishra, Kumari, & Jha, 2011). MALDI TOF–TOF mass spectroscopy of biosurfactant (S3) represented mass peaks m/z 156.0789 and 175.1147 corresponding to pentose and hexose sugars respectively. A series of masses m/z 303.1172, 330.0684 and 359.0809 correspond to mass of disaccharides (2 pentose, 1 pentose + 1 hexose and 2 hexose respectively). Besides this, masses m/z 430.1235, 448.9248, 480.1520 and 504.2340 were also detected in positive ion reflector mode, assigned to trisaccharides however higher mass peaks m/z ranging 574.2108–1198.3400 (S4) revealed the presence of oligosaccharides chain consist of different ratios of pentose and hexose sugars.

3.6. FT-IR and NMR analyses

The FT-IR spectrum of lyophilized biosurfactant (Fig. 2) showed a strong peak at 3416 cm^{-1} which was attributed to the stretching vibration of O–H bond and the peak at 2929 cm^{-1} was associated with the stretching vibration of C–H bond of constituent sugar residues. The peak at around 2107 cm^{-1} also indicated aliphatic C–H bonds. A sharp peak at 1645 cm^{-1} suggested the presence of carbonyl functionality present in carboxylate or amide moieties of protein and peptide amines. Futhermore, the additional peak at 1078 cm^{-1} (cyclic C–O) implied the presence of uronic acid and *O*-acetyl ester. The strong absorption at 1251 cm^{-1} revealed the presence of sulfate groups as S=O and C–O–S (Parikh & Madamwar, 2006). The broad stretch of C–O–C, C–O at 1000–1200 cm^{-1} exhibited the presence of carbohydrates. The prominent absorptions at 1149 cm^{-1} and 1078 cm^{-1} showed the presence of α-pyranose form of the glucosyl residue and PII band polysaccharides. The peak at 851 cm^{-1} was attributed to glycosidic linkages. A number of peaks (signals) were observed in ^1H NMR due to the presence of different sugars moities, protein content and sulfated sugars, etc. The ^1H NMR spectra seems to be quite complex, however, some distinguished peaks were present which are described as follows. The presence of chemical shift (δ) value at 1.11–1.20 ppm indicated the methyl group ($-CH_3$) corresponding to the sugar moiety. The δ value at 1.88 ppm showed the CH-proton (of C_5/C_6 carbon of deoxy sugar). The presence of sufate group (sulfate derivative of biopolymer) was proved from the δ value 2.13 ppm. The number of anomeric signals of sugar was observed in the region 2.97–3.97 ppm of ^1H NMR spectrum. The presence of NH_2 group of protein of biosurfactant was confirmed from the δ value of 5.25–5.37 ppm (Fig. 3). Similar characteristic spectral peaks of NMR were also observed in biopolymers obtained from different sources (Mishra & Jha, 2009; Singh et al., 2011).

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

Thermal stability of biosurfactant is an important characteristic for its commercial utilization. Degradation of biosurfactant took place by two well differentiated steps as observed in TG analysis (Fig. 4a). Eleven percent (11.49%) of weight loss was recorded from 30 to 110 °C due to loss of alcohol and moisture molecules followed by second phase degradation at 280 °C, where a weight loss of 51.44% was observed with maximum degradation at 300 °C. Complete loss of biosurfactant was observed after 400 °C. The present study of TGA is different from previously reported microbial glucan produced by *Geobacillus tepidamans* V264 biopolymer, which was stable up to 250 °C with maximum degradation at 280 °C (Kambourova, Mandeva, Dimova, Poli, & Nicolaus, 2009). The DSC thermogram showed exothermic peak of biosurfactant (Fig. 4b) with crystallization temperature (T_c) of 117.11 °C (onset temperature 111.76 °C) accompanied with 198.06 J latent energy. The melting peaks were found at 178.77 °C (onset temperature 178.59 °C) with 3.07 J latent energy for T_{m1} and 242.77 °C (onset temperature 244.05 °C) with latent energy 26.52 J for T_{m2}. The activation energy (nth order of reaction) of exothermic transitions was 89.86 ± 0.13, 475.39 ± 2.91 and 192.11 ± 1.38 kJ/mol for T_c, T_{m1} and T_{m2} respectively.

3.8. Particle size and EDX analysis

The biosurfactant was composed of particles with sizes ranging from 0.538 ($d_{0.1}$) to 38.45 ($d_{0.9}$) μm with an average size of 1.155 ($d_{0.5}$) μm and specific surface area of 5.5665 $m^2\ g^{-1}$ (S5). The qualitative elemental analysis was performed by SEM-EDX (S6) which revealed the weight and atomic percentage of different elements present (C, O, Na, S, and Ca) in the biosurfactant produced by alkaliphilic bacterium RJ-06. The presence of sulfate, acetyl and carboxyl functional groups in the biopolymer proved its anionic nature

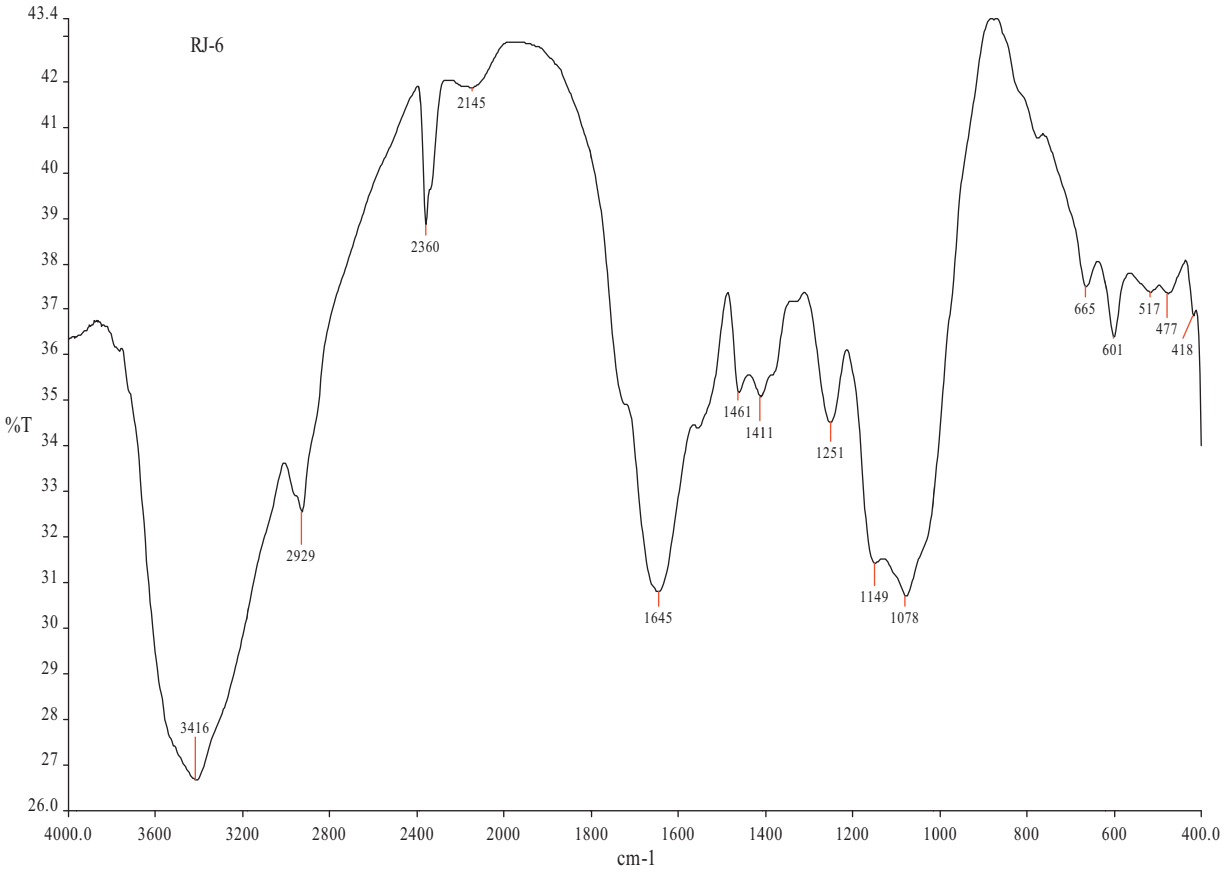

Fig. 2. FT-IR spectrum of biosurfactant extracted from alkaliphilic bacterium RJ-06.

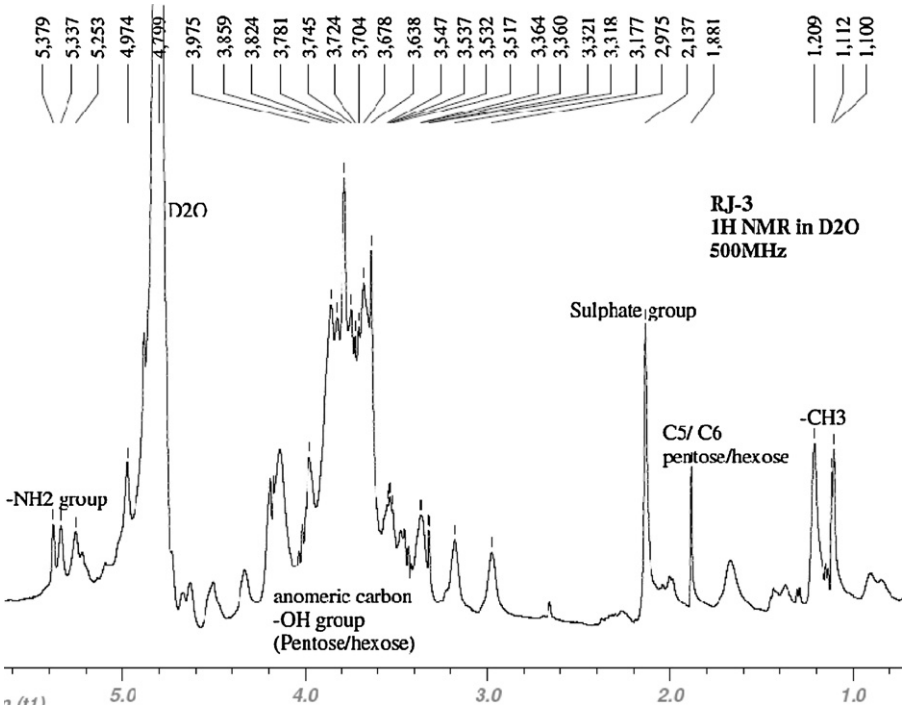

Fig. 3. [1]H NMR spectrum of biosurfactant produced by alkaliphilic bacterium RJ-06.

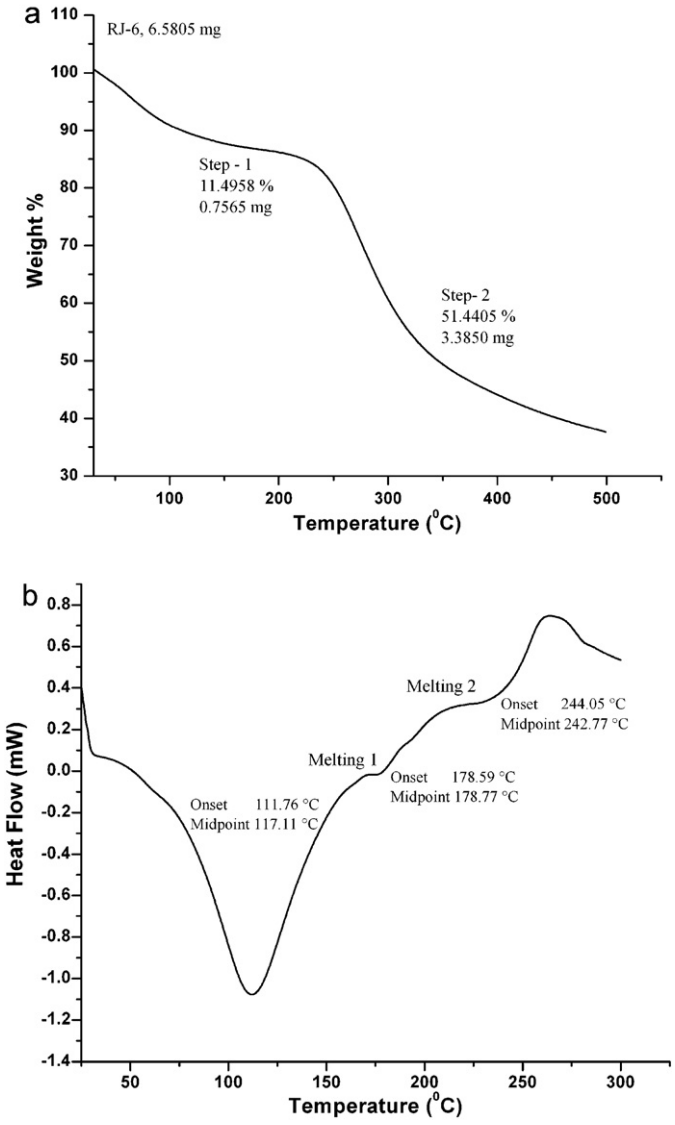

Fig. 4. TG (a) and DSC (b) thermograms of biosurfactant obtained from alkaliphilic bacterium RJ-06.

which could be useful for binding and thus remediating divalent cations (Bramhachari et al., 2007; Singh et al., 2011; Sutherland, 2001).

3.9. Identification of biosurfactant producing strain RJ-06

The bacterium used in the present study was identified as *C. sakazakii* based on 16S rRNA gene sequence homology. The strain RJ-06 was found to have closest proximity with *C. sakazakii* (EF059857) with 100% query coverage and 99% homology (Iversen et al., 2007). The 16S rRNA gene sequence has been deposited in NCBI Genbank with the accession no. JN398668.

4. Conclusion

Biosurfactants are ecologically accepted, low toxic, biodegradable and effective in a wide range of extreme conditions including temperature, pH and salinity compared to chemical surfactants. The biosurfactant produced by an alkaliphilic bacterium, *C. sakazakii*, is a heteropolysaccharide–protein complex comprised of glucose, galactose, mannose, xylose, arabinose and uronic acid. FT-IR and NMR spectrum indicated the presence of carboxyl, acetyl, sulfate and amino functional groups. MALDI TOF–TOF exhibited mass peaks m/z corresponding to oligosaccharide, constituted of galactose, glucose, xylose, mannose and arabinose, supporting GC–MS analysis.

Isolated biosurfactant showed pseudoplastic rheology and the viscosity was decreased concomitantly with an increase in the shear rate. The shear thinning behavior of the biosurfactant is useful for biotechnological applications. Extracted biosurfactant has sulfate, acetyl and carboxyl functional groups and presence of these in the biopolymer proved its anionic nature which is useful for binding of cations and thus for its remediation. The extracted biosurfactant efficiently emulsified aliphatic, aromatic hydrocarbons and oils. It had a vital property of dissolving readily in water having a broad pH range. The uronic acid and proteinaceous component of bacterial biosurfactant played an important role in the emulsification of hydrocarbons and oils, apart from functional groups (acetyl) present in the biopolymer, which also provide hydrophobicity, imparting enhanced emulsifying activity. The stable emulsifying activity makes this biosurfactant as an alternative to the commercially available chemical surfactants/emulsifiers. The thermostability and a wide range of pH tolerance with pseudoplastic behavior, makes this biosurfactant a promising candidate for biotechnological and industrial applications.

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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.2011.10.065.

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