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Exopolysaccharide from psychrotrophic Arctic glacier soil bacterium *Flavobacterium* sp. ASB 3-3 and its potential applications†

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A novel exopolysaccharide (EPS) producing psychrotrophic bacterium *Flavobacterium* sp. ASB 3-3 was isolated from Arctic glacier soil and identified. The optimum fermentation conditions for EPS production were an initial medium pH of 7.2 and an initial inoculum size of 5% (v/v). The maximum yield of EPS (7.25 ± 0.26 g L^{-1}) was obtained after cultivation at 25 °C for 120 h with glycerol as the sole carbon source. The EPS was purified and its structural characteristics were analyzed by ^{1}H and ^{13}C NMR. The predominant repeating units of this EPS are (α, β) D-glucose and D-galactose and it is different from the structure of EPSs produced by other Arctic and Antarctic bacteria, which have mannose units. In addition, EPS has demonstrated a comparable emulsifying property than SDS and flocculating properties with kaolinite, suggesting their potential applications in various industries. The EPS also significantly improved the tolerance of *Flavobacterium* sp. and *Escherichia coli* from freeze–thaw cycles, suggesting that it might be used to survive in polar regions and it can have possible usage as microbial cryoprotectants.

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1. Introduction

The increased demand of natural polysaccharides for industrial applications has led to increased consideration of bacterial exopolysaccharides (EPSs). The bacterial EPSs are long-chain branched hetero- or/homo-polysaccharides comprising repeated units of the monosaccharide moieties which are synthesized and secreted by bacteria when in the presence of an excess of the carbon source in the growth medium or under stressful environments.[1–4] Due to their physical and chemical properties, the bacterial EPSs are widely used in the different industrial sectors as bioflocculants, bioabsorbants, stabilizers, emulsifiers, drug carriers, ion exchange resins and thickening agents.[5–7] The EPSs are also used in medical fields as anti-tumor, anti-viral and anti-inflammatory agents.[8,9] At present, there has been an increasing interest in search of novel EPSs and few of them are being currently marketed as commercial products, and include xanthan, alginates, cellulose, pullulan, gellan, hyaluronic acid and succinoglycan from different bacterial strains.[1,4,5]

Emulsifiers and flocculants have been used extensively in almost every industrial sector of modern industry today.[10] A large fraction of emulsifiers and flocculants are produced by chemical synthesis and this raises concern over their potential toxicological effects to the natural environment. Because of the limitations of these chemical emulsifiers and flocculants, biopolymers produced by various microorganisms are being investigated as alternative emulsifiers and flocculants.[4,5,11] Biopolymers are biodegradable and their intermediates and by-products are harmless towards human beings and the environment. In addition, microbial derived polymers can exhibit enhanced performance and greater functional diversity than synthetic polymers.[5,12] It has been reported that many bacteria are able to produce EPS based emulsifying and flocculating agents for enhanced oil recovery/or hydrocarbon degradation and waste-water treatment, respectively.[2,3,5,13,14] Hence, high molecular weight EPSs with emulsifying and flocculating properties are of particular interest for various biotechnological and industrial sectors.

The production of EPS is an exclusive metabolic process as different polysaccharides with unique functional properties can be produced from different strains of the same species.[7,9] Due to

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this, vast numbers of microbial strains are being evaluated to find novel EPSs for commercial applications.[4] Currently, Arctic and Antarctic polar bacteria are recognized as a rich source of biological macromolecules that are of potential interest towards various industrial applications,[15] but to date, only very few reports are available on EPS from polar bacteria and their industrial prospects.[15–20] In the polar environment, bacterial EPSs are essential for the formation of aggregates, adhesion to or colonization of surfaces, formation of biofilms and possibly acting as ligands for trace metals sequestration (nutrients) or providing cryoprotection for growth at low temperature and high salinity.[3,15,18] This wide spectrum of functional diversity is reflected not merely in the complex chemistry of these carbohydrate polymers but also in the diversity of bacterial genera found producing them.[3,15,18] Therefore, it is presumed that extensive research on polar bacteria would provide a prominent source of commercial EPSs with sustainable properties. To our knowledge, this would be the first report on the production of EPS from psychrotrophic Arctic soil bacterium to explore their industrial and ecological implications. The present study was aimed to isolate and characterize the EPS producing novel strains from Arctic soil especially nearby moving glaciers, and this is followed by the production and structural characterization of a novel EPS from psychrotrophic Arctic strain *Flavobacterium* sp. ASB 3-3. The possible ecological roles of the EPS for the adaptation of the strain ASB 3-3 in the extreme polar environment are discussed. Further, the produced Arctic EPS was evaluated for their emulsifying, flocculating and cryoprotective efficacy to explore their promising biotechnological applications.

2. Experimental section

2.1. Isolation and screening of EPS-producing bacteria

The Arctic soil samples were collected from Midtre Lovenbreen which is located close to Ny-Ålesund in Svalbard, during on July 2014. The samples were collected from 12 different sites (AS-01 to AS-12) at 0–5 cm depth and the sampling sites were represented in Fig. S1.† The samples were immediately frozen in dry ice and then stored at −80 °C until they were processed for bacterial enrichment and isolation. The permissions were obtained for these location/activities (Rig no.: 6752, environmental change studies based on the Arctic Dasan station: in terms of Geology, Atmospheric Science, and Ecology). For enrichment, one gram of collected soil sample was inoculated into 10 mL of nutrient broth (Acumedia) and 10 mL of Luria Bertani (LB) broth (Merck KGaA). The sample inoculated Erlenmeyer flasks were incubated at 20 °C for 24 h. The enriched culture was then serially diluted and plated on Nutrient Agar (NA), Tryptic Soy Agar (TSA) and Luria Bertani Agar. Agar plates, which were incubated at 4, 15, 20 and 25 °C for two weeks. Isolates displaying distinct colony morphologies were subcultured onto their respective growth medium and stored in LB broth supplemented with 20% glycerol. The EPS-producing isolates were screened by using nitrogen-deficient nutrient medium[4] and the composition of screening medium included sucrose, 20 g L^{-1}; NH_4NO_3, 1.0 g L^{-1}; yeast extract, 0.1 g L^{-1}; KH_2PO_4, 0.3 g L^{-1}; K_2HPO_4, 0.3 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1}; $MnSO_4 \cdot 4H_2O$, 0.1 g L^{-1}; $CaCO_3$, 0.4 g L^{-1}; NaCl, 0.05 g L^{-1} and tryptone, 0.1 g L^{-1} with the initial pH 7.0 ± 0.2. Arctic strains were inoculated into 100 mL Erlenmeyer flasks containing 20 mL of screening medium and incubated in a shaker at 150 rpm for 72 h at 25 °C. After incubation, the cell-free supernatant (CFS) was collected and checked for EPS production by ethanol precipitation and phenol sulfuric acid assay. EPS producers were grown in a 2% glycerol enriched medium to confirm their productivity and compared with reference polar bacterial strains and other known EPS producers to select the hyper-EPS producing Arctic strain. Strain ASB 3-3, which had the highest EPS production, was chosen for further study. All chemicals used in this study were of analytical grade.

2.2. Identification of EPS-producing strain ASB 3-3

The morphological and biochemical characteristics of the Arctic strain ASB 3-3 were identified according to Bergeys's Manual of Determinative Bacteriology.[21] For molecular characterization, the genomic DNA was extracted from 2 mL of pure culture of ASB 3-3 and nearly full-length 16S rRNA gene was amplified by using universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′).[22] A 25 μL reaction volume PCR was performed using about 10 ng of genomic DNA, 1× reaction buffer (10 mM Tris–HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100), 0.4 mM (each) deoxynucleoside triphosphates (Invitrogen) and 0.5 U of DNA polymerase (New England Labs, UK) and 1 mM each forward and reverse primers. The PCR temperature profile used was thus 95 °C for 3 min, then 30 cycles consisting of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1.45 min and finally an extension step at 72 °C for 10 min. The PCR amplicon was cloned by the TA cloning method using TOPO TA Cloning kit according to manufacturer's instructions (Invitrogen) for sequencing. 16S rRNA gene sequences obtained from the isolate ASB 3-3 was compared with other bacterial sequences by using NCBI Mega BLAST (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) for their pairwise identities. Multiple alignments of these sequences were carried out by ClustalW2 version of EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with 0.5 transition weight. Phylogenetic trees were constructed in MEGA 6.06 version (http://www.megasoftware.nte) using neighbor joining (NJ) and unweighted pair-group method with arithmetic mean (UPGMA) algorithm. The partial sequences of the 16S rRNA gene of strain ASB 3-3 were submitted and registered in GenBank with the accession number KT276370.

2.3. Production, extraction and purification of EPS from strain ASB 3-3

The Arctic strain ASB 3-3 was inoculated into culture medium containing glucose, 10 g L^{-1}; yeast extract, 0.5 g L^{-1}; KH_2PO_4, 0.2 g L^{-1}; NaCl, 0.1 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1} in order to prepare the inoculum for the batch fermentation process. The initial production of the EPS was performed in 1000 mL Erlenmeyer flasks containing 500 mL of production medium with 25 mL of seed culture. The composition of the production

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medium was as follows: glycerol, 30 g L^{-1}; yeast extract, 0.5 g L^{-1}; $NH_4H_2PO_4$, 3.3 g L^{-1}; KH_2PO_4, 3.7 g L^{-1}; K_2HPO_4, 5.8 g L^{-1}; $MgSO_4 \cdot 7H_2O$ (100 mM), 10 mL L^{-1} and micronutrient solution (MNS), 1 mL L^{-1}. The MNS comprises $FeSO_4 \cdot 7H_2O$, 2.18 g L^{-1}; $MnCl_2 \cdot 4H_2O$, 1.98 g L^{-1}; $CaCl_2 \cdot 2H_2O$, 1.67 g L^{-1}; $CuCl_2 \cdot 2H_2O$, 0.17 g L^{-1} and $ZnSO_4 \cdot 7H_2O$, 0.29 g L^{-1}. The initial pH of the medium was adjusted to 7.0 ± 0.2. The inoculated flasks were incubated with 150 rpm agitation at 25 °C for 7 days. All experiments were performed in triplicate. After incubation, the culture volume of 1 L was pooled and centrifuged at 12 000 × *g* for 30 min at 4 °C and the cell pellet (Biomass) was lyophilized and weighed. The EPS extraction was performed according to literatures.[4,23] The cell-free supernatant (CFS) was subjected to thermal treatment (80 °C, 1 h) to inactivate bacterial enzymes that might cause EPS degradation during the following EPS precipitation and purification steps. The CFS was concentrated into 100 mL using a rotary evaporator (Eyela world, Tokyo Rikakikai Co., Ltd). For the extraction and purification of EPS, three volumes of ice-cold absolute ethanol was added into concentrated CFS in a 500 mL Erlenmeyer flask and kept at 4 °C for overnight for the EPS precipitation. The exopolymeric precipitate was collected by high-speed centrifugation at 14 000 × *g* for 30 min at 4 °C. The precipitate was washed with 70–100% ethanol–water mixtures. After ethanol–water washing three times, the precipitates were pooled and dried in a desiccator and stored at room temperature till needed. The EPS precipitate was dissolved in deionized water and its protein content (impurities) were removed (deproteinization) by trichloroacetic acid and the Savag method.[16] The deproteinization was repeated twice and the extracted EPS were combined and redissolved in deionized water and dialyzed at 4 °C for 12 h against deionized water for desalting and to remove the salt impurities. Excessive water content was removed under vacuum and then the sample was lyophilized. The purified EPS was stored at room temperature until physical and chemical analyses were performed.

2.4. Chemical and structural characterization of the EPS

2.4.1. Chemical analysis. The lyophilized crude EPS was dissolved in ultrapure Milli-Q water (0.1 g L^{-1}) and the total carbohydrate contents were assayed by phenol sulfuric acid method with glucose as standard.[24] The total protein content was determined with bovine serum albumin (BSA) as standard using Bradford assay.[25] Sulfated sugars were calculated by measuring the amount of sulfate content using K_2SO_4 as standard.[26] The chemical composition of the crude and deproteinized EPS was investigated by thin layer chromatography (TLC). The control tests were performed with commercial sugars as standards for the identification of sugar composition in the bacterial EPS.

2.4.2. High-performance liquid chromatography (HPLC). The deproteinized EPS sample (2–3 mg) was dissolved in 2 mL deionized water and hydrolyzed with 0.1 mL of 99% trifluoroacetic acid (TFA) at 120 °C for 120 min. The hydrolyzed EPS was used for the identification of monosaccharides present in the purified EPS. The monosaccharide analysis was performed by HPLC with an Aminex HPX-87H column (BioRad), coupled to an ultraviolet (UV at 210 nm) and refractive index (RI) detector, using 5 mM sulfuric acid (H_2SO_4) as eluent, at a flow rate of 0.600 mL min^{-1} with oven temperature of 50 °C.

2.4.3. Fourier-transform infrared (FT-IR) spectrometry. FTIR Spectroscopy (Tensor 27, Bruker Corporation) was used to analyze the major functional groups which are present in the purified EPS of Arctic strain ASB 3-3. The sample pellets were prepared by mixing the lyophilized EPS (0.75 mg) with 300 mg of potassium bromide (KBr). Double sided FT-IR spectra were acquired with a resolution of 4 cm^{-1} in 4000–600 cm^{-1} region. Spectra were corrected for wavenumber-dependent signal detection efficiency of the setup using the white light spectrum of a temperature-calibrated tungsten band lamp.

2.4.4. ^{13}C Solid-state NMR spectrometer. Solid-state nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance II 500 MHz spectrometer (Bruker Co., Billerica, MA) with a 5 mm pulsed field gradient (diffusion) probe. The spectra were run in the temperature range −50 to 80 °C. The purified EPS of the strain ASB 3-3 was dissolved in D_2O at concentrations of 5 mg mL^{-1} (for ^1H NMR) and 20 mg mL^{-1} (for ^{13}C NMR).

2.5. Emulsification activity of the EPS

Emulsification activity of 1% EPS purified from Arctic strain ASB 3-3 was checked with *n*-hexane. *n*-Hexane (95%, Samchun) was added to EPS solution (5%) in a ratio of 1 : 1 and manually shaken, vortexed at 2000 rpm (2 min) and left over for 10 min. The height of the emulsification layer (emulsification index [EI_{24}]) was measured after 24 h incubation at 25 °C. The emulsification index (EI_{24}) was calculated using a formula:

$$\text{Emulsification index } (EI_{24}) = \frac{H_{EL}}{H_S} \times 100\% \quad (1)$$

where, H_{EL} is the height of the emulsified layer and H_S is the height of the total liquid column. Sterile distilled water was used as the negative control.[11] This same assay was also used to measure the EI_{24} produced by the solutions of the extracted EPS against hydrocarbons such as *n*-hexadecane (99%, Alfa Aesar), methyl octanoate (99%, Aldrich), methyl 10-undecanoate (96%, Aldrich), toluene (99%, Samchun) and petroleum (~18%, Sigma-Aldrich). Commercial surfactant such as 1% sodium dodecyl sulfate (SDS) was used as positive control and compared with EPS for emulsification activity.

2.6. Determination of flocculating activity of EPS and their properties

The flocculating activity was predicted using a solution of kaolinite ($Al_2Si_2O_5(OH)_4$) as the suspended solid. Briefly, 5.0 mL of 1% (w/v) $CaCl_2$ and 0.2 mL of EPS of strain ASB 3-3 (5 mg L^{-1}) were added into 95 mL of kaolinite suspension (5.0 g L^{-1}, pH 8.0). The mixture was stirred for 4 min and then allowed to incubate for 5 min at 28 °C. The optical density (OD) of the aqueous phase was measured at 550 nm with a UV/visible spectrophotometer (Amersham Biosciences). A control was prepared in the same way, except in the absence of EPS, and the

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flocculating activity was calculated based to the following mathematical equation.

$$\text{Flocculating activity} = \frac{B - A}{A} \times 100\% \quad (2)$$

where A and B are the OD of the EPS and the control, respectively. The effects of EPS concentration, temperature and pH of the solution on flocculating activity were also examined. The concentration of EPS varied from 1–8 mg L^{-1}. The pH of the kaolinite suspension was adjusted using 1 M NaOH and 1 M HCl in the pH range of 5–12.0. The temperature of kaolinite suspension was changed in a water-bath in the range of 5–60 °C.

2.7. Analysis of cryoprotective effect of the EPS

To examine the cryoprotective activity of the purified EPS, *Flavobacterium* sp. strain ASB 3-3 and *E. coli* DH5α were exposed to multiple freeze–thaw cycles in the presence or absence of the EPS. Strain ASB 3-3 and *E. coli* DH5α were inoculated into an Erlenmeyer flask (100 mL) containing 20 mL LB broth medium and incubated for 12 h with agitation of 200 rpm at 25 and 37 °C, respectively, and until reaching late logarithmic phase (OD_{600} = 0.8). About 1.5 mL of culture was harvested by centrifugation at 8000 × *g* for 10 min at 4 °C, washed thrice with deionized water, and then resuspended with 500 μL of 0.9% (w/v) NaCl solution (physiological saline). An equal volume of EPS solutions at different concentrations (0, 5, 10, 20, 30 and 50 mg mL^{-1}) was mixed with the suspensions of strain ASB 3-3 or *E. coli* DH5α cells in 1.5 mL sterile tubes. An equal volume of 0.9% NaCl solution mixed with the suspensions of strain ASB 3-3 or *E. coli* DH5α cells was used as a control. The mixtures were frozen at −72 °C for 1 h and thawed for 30 min in a water-bath at 25 °C. The freeze–thaw cycle was continued up to 8 consecutive times. At the end of the 0, 2nd, 4th, 6th or 8th thawing, the mixture was diluted up to 10^{-5}, and then plated on LB agar to examine the number of surviving colony forming unit (CFU).

2.8. Statistical analysis

All the experiments were carried out three times. Data were expressed as mean ± SD (n = 3). Statistical analysis of the experimental data was carried out by MS Excel 2010 and sigma plot.

3. Results and discussion

3.1. Screening and identification of Arctic strain ASB 3-3

A total of 53 Arctic strains were screened for the production of EPS from nitrogen-deficient medium amended with sucrose (data not shown). Six strains were shown to produce EPS under nitrogen-deficient sucrose medium and further EPS productivity was confirmed by using 2% glycerol medium. The EPS producing polar strains were compared with reference polar strains and other known EPS producers. The Arctic polar strains were shown to exhibit more significant EPS productivity than other reference strains. The EPS yields of those strains under our experimental conditions were in the range of 0.41 ± 0.09 to 6.24 ± 0.73 g L^{-1} (Fig. 1). Strain ASB 3-3 grew well in both sucrose and glycerol enriched medium, which exhibits their maximum EPS productivity (6.24 ± 0.73 g L^{-1}) when the production medium was supplemented with 2% glycerol (wt/vol). During the initial screening, reference strains *Pseudomonas* sp. PAMC 22752 and *Pseudomonas olevarens* were shown to have moderate productivity, which was less than for strain ASB 3-3.

The active EPS producing strain ASB 3-3 was identified by morphological, biochemical characteristics and 16S rRNA based phylogenetic analysis. Microbiological properties were compared with Bergey's manual of determinative bacteriology (Table S1†). On the basis of the amplified sequence of the 16S rRNA gene of strain ASB 3-3, the phylogenetic relationship of this strain was determined. Taxonomic affiliation of the 16S rRNA sequences of the strain ASB 3-3 was retrieved from classifier program of Ribosomal Database Project II release 11.4 (http://www.rdp.cme.msu.edu/). The 16S rRNA sequence of the isolate was blasted using megablast tool of GenBank (http://www.ncbi.nlm.nih.gov/). Thus, on the basis of 16S rDNA sequence and phylogenetic relatedness, it was revealed that the organism was *Flavobacterium*, for which many Arctic and Antarctic strains are found.[18] Representative maximum homologous (98–99%) sequences of each isolate were obtained from the seqmatch program of RDPII and were used for the construction of UPGMA phylogenetic affiliation (Fig. S2†). The Arctic isolate ASB 3-3 showed a unique cluster between *Flavobacterium pectinovorum* AD-R2 (KF704086) and uncultured *Flavobacterium* sp. clone SNNP 2012-65 (JX114398) (Fig. 2). Therefore, on the basis of morphological, biochemical, physiological, and analysis of the 16S rRNA gene sequence, the newly isolated Arctic strain ASB 3-3 was designated as *Flavobacterium* sp. and deposited into Polar and Alpine Microbial Collection (PAMC), Korea Polar Research Institute under the accession number PAMC 28614.

Studies on the diversity of Arctic and Antarctic polar microbial communities have showed that the *Gammaproteobacteria* is a predominant taxonomic group usually found in these extreme environments, as determined by both cultivation-dependent and cultivation-independent approaches.[18,27] *Flavobacterium*, a genus of the *Gammaproteobacteria* under *Flavobacteriaceae* family, is a universal commensal bacteria that live in soil and water, and a few of them are opportunistic pathogens.[28] In this study, strain ASB 3-3, which produces the high amount of EPS, was screened from 53 Arctic soil isolates and was identified to be a member of the genus *Flavobacterium*. There are very few studies about the *Flavobacterium* spp. especially from polar environment and it has the great potential in the bioprospecting aspects for novel biological macromolecules.[18] The strain ASB 3-3 isolated from Ny-Alesund, Midtre Lovenbreen, exhibited the psychrotrophic growth pattern and our results corroborates with previous studies about the existence of the psychrotrophic nature of *Flavobacterium* in natural environments.[28–30] To best of our knowledge this is the first report on the EPS from an Arctic glacier soil psychrotrophic bacterium.

3.2. EPS production and growth characteristics of strain ASB 3-3

Strain ASB 3-3 grew very slowly in both solid and liquid media, meanwhile it was isolated from the Arctic polar region, but after

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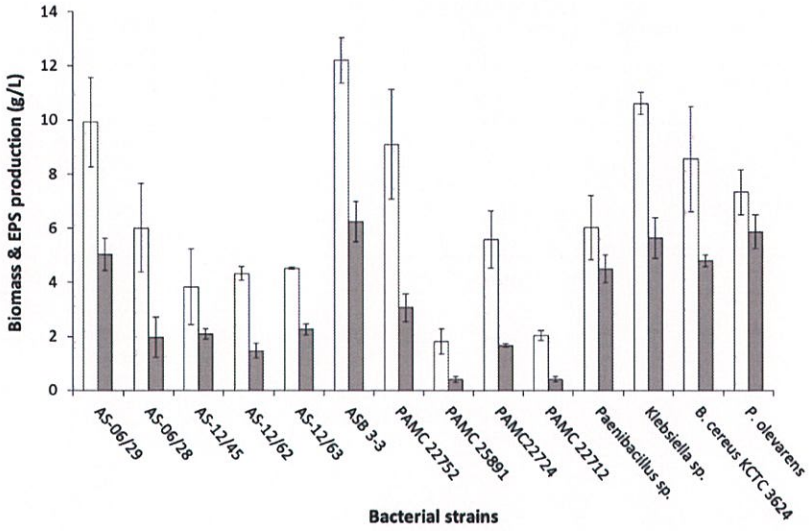

Fig. 1 EPS production from six Arctic strains (AS & ASB), four polar strains (PAMC) and four other reference strains in 3% glycerol enriched medium. EPS production was determined by quantifying the whole EPS content after lyophilization. The values shown are means ± SDs from three experimental replicates. White bars, biomass (g L^{-1}); dark grey bars, EPS production (g L^{-1}).

5 days incubation in liquid media at 25 °C, robust growth was observed and then it was termed as a psychrotrophic bacterium. Fig. 3 shows the growth of strain ASB 3-3 in production media amended with 3% glycerol and production of its EPS. Exponential growth commenced immediately after inoculation, reaching a maximum cell density ($OD_{595\ nm}$, 3.86 ± 0.17) at 144 h of cultivation in shake flask culture. The EPS synthesis was started at the early log phase (24 h) and continued up to 120 h of incubation. The maximum synthesis of EPS was reached at early stationary phase of growth and it was slightly decreased after

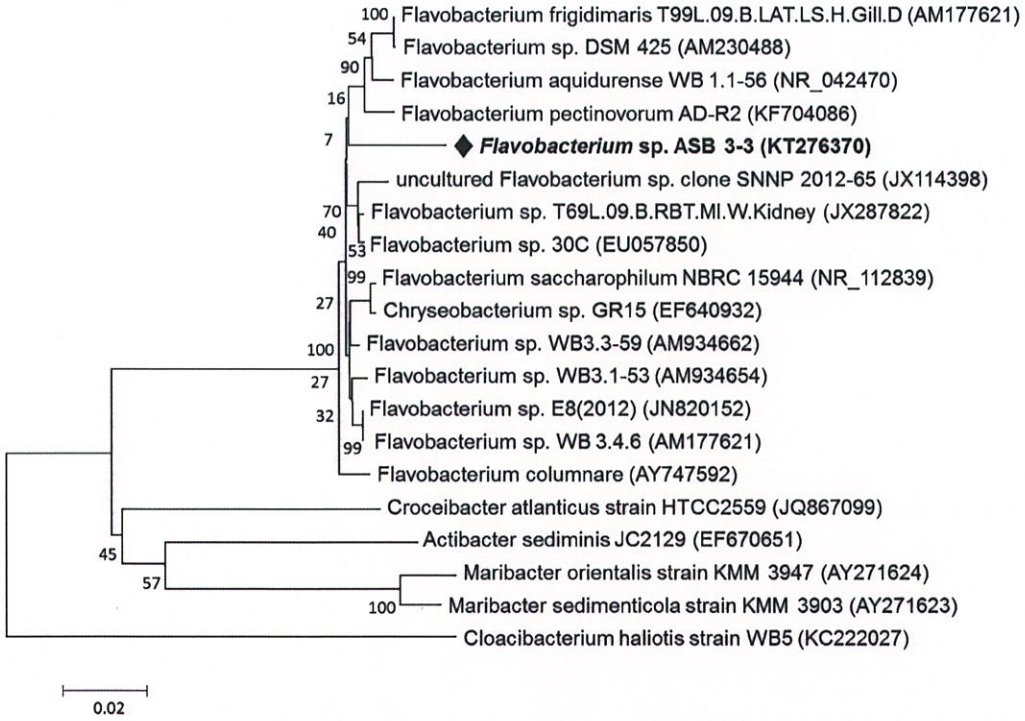

Fig. 2 Neighbor-Joining (NJ) bootstrapping (1000) phylogenetic tree of Arctic soil bacterium ASB 3-3 and their closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences. Phylogenetic tree was developed based on maximum composite likelihood method using MEGA 6.06 version.

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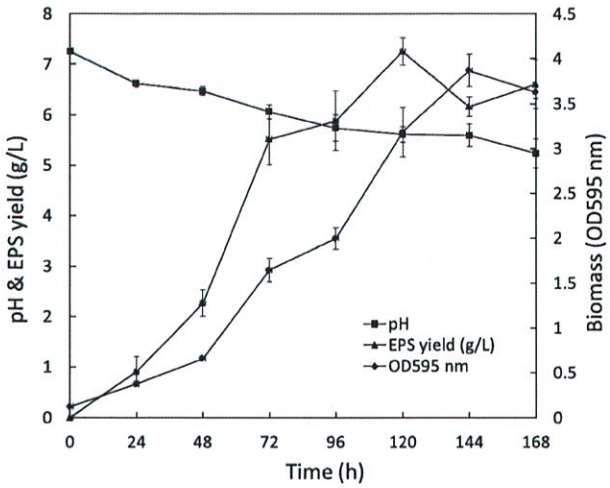

Fig. 3 Growth curve of strain ASB 3-3, pH change and EPS production. Strain ASB 3-3 was cultivated in 3% glycerol medium. Error bars indicate the SD.

the entering into stationary phase and also EPS productivity was increased in nitrogen source limiting condition. The increase of EPS synthesis was coupled to an almost proportional decrease in the measured pH of the production medium, from an initial value of 7.2 ± 0.02 to 5.6 ± 0.15 after 120 h. The EPS synthesis was confirmed by phenol sulfuric acid assay and assessed by dry weight of the extracted polymer. The maximum EPS productivity of about 7.25 ± 0.26 g L^{-1} was achieved with biomass of 3.18 ± 0.27 ($OD_{595\ nm}$) from the Arctic strain ASB 3-3. The EPS production in bacteria isolated from cold environments including Arctic and Antarctic regions was compared (Table 1) and the strain ASB 3-3 was found to produce the highest amount of EPS among the polar strains.

Flavobacterium is a slow growing bacterium[31] as was also observed in this study. Growth of strain ASB 3-3, upon inoculation, quickly entered into the log phase without undergoing an obvious lag phase when inoculated at ratio of 1 : 100 (v/v). The bacterium reaches the stationary phase after 144 h of incubation and the maximum EPS productivity was reached before attaining the stationary phase of the growth. Absence of the lag phase and arrival at the maximum yield of EPS in the early stationary phase would be ideal for economic production of EPS. It was observed that the EPS productivity ran parallel to cell growth, thus indicating a concomitant increase in EPS production with cell growth. This observation indicated that the production of EPS was a result of biosynthesis during the bacterial growth and not by nutrient starvation or cell autolysis.[14] The decrease on EPS productivity observed after 120 h could be attributed to the accumulation of EPS-degrading enzymes produced by the bacterium.[32] A similar observation was also reported with other microorganisms such as EPS-producing lactic-acid bacteria and halophilic bacterium *Halomonas almeriensis*,[8,33] since EPS yields increased during the log phase and stopped when the stationary growth phase was reached.[2] These results do not agree with those of other authors, however, who maintain that cell growth and EPS formation usually have different nutritional requirements.[9,13] It was observed that there was a decrease in pH of the production medium as cultivation time progresses. The decrease in pH of the medium may be due to the production of organic acids as a result of glycerol metabolism since glycerol was a sole carbon source of the production medium, or the decrease in pH might be due to the presence of organic acids produced during metabolism by bacteria.[14,32]

3.3. Chemical and structural characterization of EPS

3.3.1. EPS chemical composition. The chemical analysis of crude EPS from ASB 3-3 revealed a gross variation in chemical composition. The contents of total carbohydrates, protein and sulfate or salts were found to be 56, 23 and 21%, respectively. In general, carbohydrate contents of the EPSs were higher than proteins and sulfated content, which is a typical feature of bacterial EPSs. The preliminary TLC experiments showed that the crude EPS contains both carbohydrate and protein parts. After the deproteinization (Sevag method) and desalting (dialysis) processes completely removed the proteins and salts, respectively, this results in high molecular weight 100% pure EPS (Fig. S3A†). The purified and deproteinized EPS was subjected for acid hydrolysis and the sugar monomeric peaks were detected from the hydrolysed EPS sample on the analytical HPLC system (Fig. S3B†). Two main constituent sugar residues were identified by HPLC analysis, namely, glucose and galactose with retention time of 13.66 and 16.05 min, respectively. Glucose was the most abundant monosaccharide accounting for 70 mol% of the total carbohydrate content of the EPS and remaining galactose represented about 30 mol%. Phenol sulfuric acid method was periodically performed along with HPLC analysis (data not shown), which are provides clear evidence for the purity of EPS.

Table 1 EPS production in bacteria isolated from cold environments including Arctic and Antarctic regions[a]

Microorganism	Isolated from	Carbon source	EPS productivity (g L^{-1})	Reference
Pseudoalteromonas sp. CAM025	Antarctic marine sea ice, Aurora Australis	Glucose (3%)	100 mg per cdw	17
Pseudoalteromonas sp. strain SM20310	Arctic sea ice, Canada Basin	Glucose (3%)	0.567	16
Pseudoalteromonas sp. SM9913	Deep sea sediment, Yellow Sea, China	Corn powder (2%) Bean powder (2%)	5.25	19
Flavobacterium sp. strain ASB 3-3	Arctic soil, Ny-Alesund, Midtre Lovenbreen	Glycerol (3%)	7.252	This study

[a] cdw: cell dry weight.

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A better understanding of the chemical and structural composition of bacterial EPSs is essential for studying their ecological roles and exploring their industrial implications. Partial chemical and structural characterizations of some EPSs produced by *Flavobacterium* have been reported,[18,34] however, there is no reports on steric structure of EPS from *Flavobacterium* spp. The results of this study showed that the EPS produced by ASB 3-3 is highly complicated and composed of two monosaccharide residues such as glucose (70%) and galactose (30%). The presence of glucose and galactose residues in microbial exopolysaccharides is rather common, even though it may be present in variable amounts.[1,4] The high glucose and galactose content of the EPS produced by *Flavobacterium* sp. grown on glycerol, distinguishes it from other bacterial EPS. Also the absence of acidic and amino sugars distinguishes it from other galactose-containing bacterial EPS.[1]

3.3.2. Functional group analysis by FT-IR spectroscopy. The FT-IR spectrum of purified EPS showed (Fig. 4A) a diverse range of absorption peaks from 3450 to 600 cm^{-1}. Presence of high levels of hydroxyl groups (O–H) stretching frequencies was identified by the broad absorption peak around 3000–3500 cm^{-1} confirming the polysaccharide nature of the purified exopolymer. Two weak absorption peaks at 2921 and 2815 cm^{-1} were assigned to C–H asymmetric stretch and C–H symmetric stretch of CH_2 and CH_3 groups, respectively. The strong absorption band at 1585 and small peak at 1618 cm^{-1} were indicative of the presence of carbonyl (C–O–H) and amide or amine (C=O, C–N) groups, respectively, in the EPS material. A strong symmetrical stretching band at 1308 cm^{-1} showed the presence of carboxyl (C=O) of –COO groups. The absorption peak at 1076 cm^{-1} corresponds to the saccharide moieties and strong absorption around 600–950 cm^{-1} was indicative of glycosidic linkage peak of polysaccharide. The infrared spectral analysis revealed characteristic peaks for carbohydrate polymers and confirmed the presence of the functional groups: carbonyl (C–O–H), hydroxyl (–OH), amide (C=O, C–N) and carboxyl (–COO), which may act as a receptor for divalent cations (Ca^{2+}) during flocculating activity.[4] Two main roles in flocculation are important characteristics of these functional groups: hydrophobicity characteristics are utilized to extend the

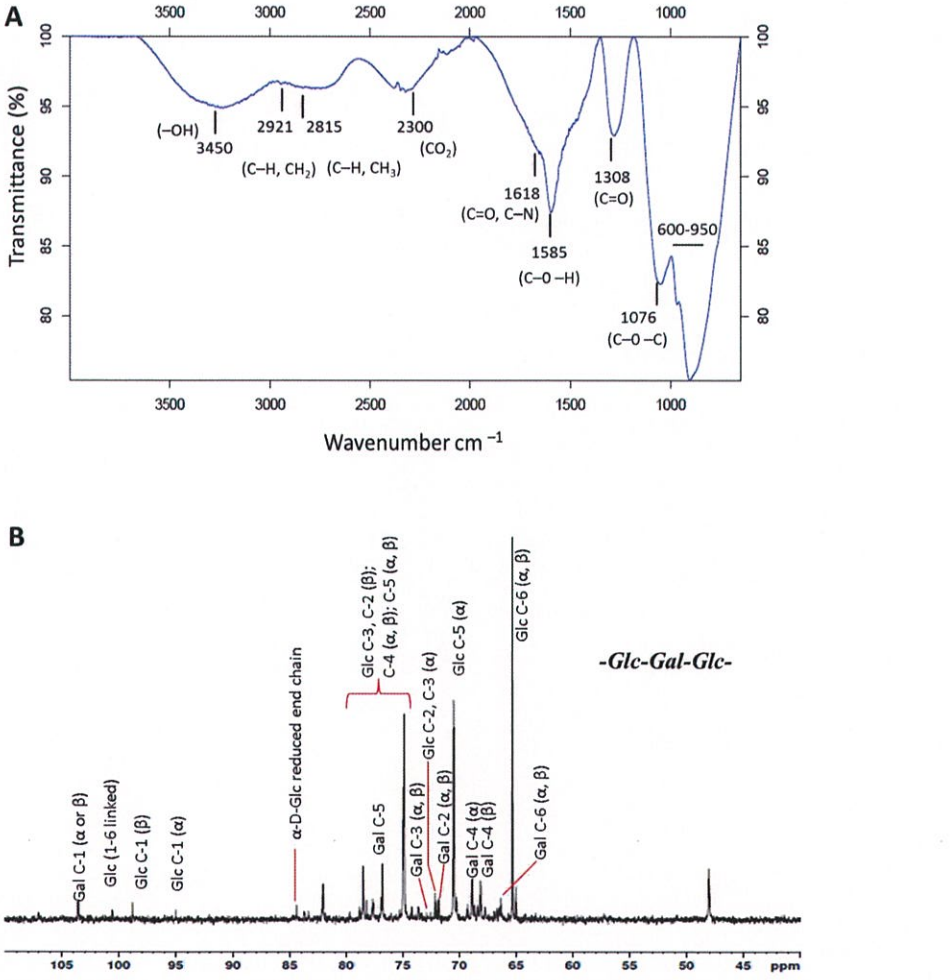

Fig. 4 (A) FTIR spectra of EPS from strain ASB 3-3, exhibiting the major functional groups. (B) Solid-state ^{13}C NMR spectra of EPS from strain ASB 3-3, revealing the presence of monosaccharides, glucose (Glc) and galactose (Gal).

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polymer chain; and the functional groups extend the gap between the chains to allow adsorption of organic particles.[12] A stretching band around 1600 cm^{-1} in the EPS of *Pseudomonas olevarens* NRRL B-14682, can be attributed to ring structure of galactose and other sugar moieties,[1] which is also observed in the EPS of ASB 3-3.

3.3.3. Structure analysis by solid-state NMR. The ^1H proton NMR spectrum showed two anomeric resonance signals at δ 4.9 and 5.2 ppm which correspond to protons of the first carbon of the monosaccharides (C-1) (Fig. S4†) and a number of overlapping signals at δ 3.6, 3.7, 3.5 and 3.8 ppm were assigned to protons (H) of the C-2, C-3, C-4 and C-5 of the carbohydrate ring regions, respectively. The two small signals at δ 3.8 and 3.81 ppm should belong to C-6 (H) of the glucose and galactose and these signals may be due to reduced end-chains. The signals at δ 4.91–4.20 ppm shifts were characteristic of glucose and galactose moieties. ^1H proton NMR spectra appear as singlets but with indications that in the same monosaccharide different locations resulted in anomeric proton shifts. Since, both glucose and galactose are epimers, for EPS in D_2O all glucose proton signals merged with other signals owing to a fast exchange in the NMR time scale.

^{13}C NMR resonances of the EPS are much more dispersed than their ^1H counterparts (Fig. 4B). The two main signals in the anomeric region occur at δ 103 (doublets as 103.50 and 103.57) and 98.77 (singlet) ppm and were assigned to C-1 position of α or β-D-galactose and β-D-glucose residues, respectively. There is a further singlet at δ 94.97 ppm and it may belong to anomeric C-1 position of α-D-glucose. A small resonance at δ 100.55 ppm was assigned to (1 $\rightarrow$ 6)-linked α-D-glucose residues. The chemical shifts around δ 70–78 ppm were assigned to C-2, C-3, C-4 and C-5 of glucose (α or β). The main peaks observed at δ 71.83, 72.18, 76.93 and 68 ppm could be ascribed to the presence of C-2, C-3, C-5 and C-4 (α or β) hydroxyl substitution of galactose. In addition, the observed δ values for the C-6 of the glucose (α or β) and galactose (α or β) (δ 66 and 65 ppm) indicated their O-6 substitution. According to the NMR results, the main components of the EPS were glucose and galactose in the form of α and β-D-glucopyranose rings connected with (1 $\rightarrow$ 6)-glycosidic linkages. From both ^1H and ^{13}C NMR, there is no background noise; hence this EPS polymer is 100% pure. The structure of this EPS is different from that of EPSs produced by other Arctic/Antarctic bacteria.[3,18] Previous reports on polar environmental bacteria showed that the mannose is a main component of many EPSs.[15,17,18] The EPS produced by *Pseudoalteromonas* spp. and *Flavobacterium* spp. isolated from polar regions, has a branched mannan structure comprised of a backbone of mannose residues.[16,18] In addition, many EPSs secreted by polar bacteria are polyanionic in nature due the presence of uronic acids, ketal-linked pyruvate, and inorganic residues.[3] In contrast, the EPS from Arctic strain *Flavobacterium* sp. ASB 3-3 have no such components and groups in their structure. Hence, a more in-depth analysis of EPSs isolated from polar environments is needed to elucidate the structure of the polymers and to elucidate the ecological roles linked with chemical composition.

3.4. Emulsification, flocculating and cryoprotective efficacy of Arctic bacterial EPS

3.4.1. Emulsification activity of the EPS. Concentrations of the purified polymer in the range 0.1–0.5% did not have any effect on the emulsification of hydrocarbons (data not shown). However, the polymer at 1% concentration was found to effectively emulsify a range of hydrocarbons. Fig. 5 shows the emulsifying activities of purified EPS of strain ASB 3-3 and the known chemical emulsifier, SDS as a control surfactant under neutral pH conditions when tested against six different hydrocarbons. The EPS exhibits a maximum emulsifying activity against *n*-hexane followed by *n*-hexadecane, with an emulsifying index of 66.33 $\pm$ 2.05 and 64.33 $\pm$ 3.29%, respectively, and it is comparable to the values observed for SDS (63.33 $\pm$ 2.49, 65.96 $\pm$ 1.23%). The purified EPS showed less emulsifying activity with methyl octanoate (26.33 $\pm$ 1.11%). Multiple one-way ANOVA exhibited that the main effect of EPS was highly significant ($P < 0.005$) (data not shown). The overall residual emulsifying activity of the EPS was estimated as 52.62 $\pm$ 14.36%, and based on the average results for all hydrocarbons, EPS produced emulsifying activities that were similar to or slightly lower than that of the control (SDS). Interestingly, even a very low concentration (1%) of EPS produced stable emulsions, which are composed of small, uniform droplets, resulting in a fine, smooth consistency, so it could well be used as an emulsifying agent in the food and oil industries, where emulsifiers from microbial sources have attracted attention because of the advantages they offer over artificial product emulsions with the various aromatic hydrocarbons. Further, the stability and high percentage of emulsions is an advantageous property both in terms of bioprocessing economics and potential industrial applications. This may be attributed to certain functional groups on the EPS polymer, such as trace amide/amine, or increased substitution by acetylated glucose/galactose, either of which can render polymeric compounds

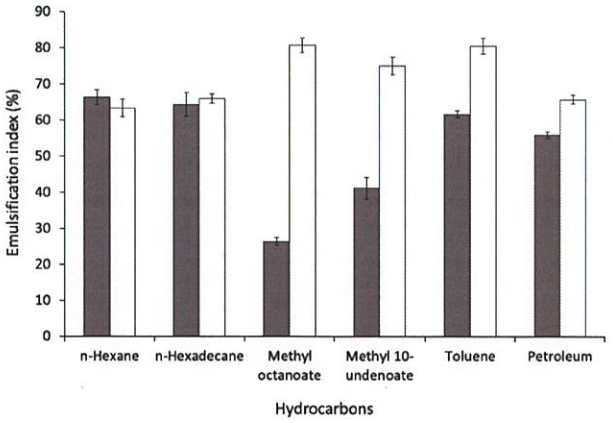

Fig. 5 Emulsification activity of the EPS purified from Arctic *Flavobacterium* sp. strain ASB 3-3. Emulsification index [EI_{24}] after 24 h was calculated with different hydrocarbons. An equal volume of sterile water in the place of EPS was used as the control. The values shown are means $\pm$ SDs from three experimental replicates. Dark grey color bars, 1% EPS from strain ASB 3-3; white color bars, 1% SDS.

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amphipathic in nature.[5,10] Carboxylate and methoxycarbonyl groups of polysaccharides have also been reported to contribute emulsifying activities to carbohydrate polymers.[7,10]

3.4.2. Flocculating activity of the EPS. An optimal concentration of EPS for flocculating activity was determined using kaolinite suspension (EPS, 20 mg L^{-1}; pH, 8.0; temperature, 25 °C). The highest flocculating activity (91.27 ± 1.01%) was observed at a concentration of 40.0 mg L^{-1} (Table 2). Around 250–300 μm flocs were formed by the EPS of strain ASB3-3 when tested for kaolin clay flocculation (data not shown). It was observed that the flocculating activity of the EPS increased as the concentration increased. Flocculation efficiency of EPS increased from 49.45 ± 9.12 to 91.27 ± 1.01% with the addition of EPS dose in the range of 5.0 to 40 mg L^{-1} EPS. Usually, low dosage will not lead to bridging flocculation mechanism of the bioflocculant (in this case EPS) to be effective and high dosage will generate high viscosity which will inhibit the settling of suspended particles by restabilization of kaolin particles.[35] The flocculating activity of the EPS from Arctic strain ASB 3-3 was directly proportional to the concentration of EPS in the solution and EPS secreted by the strain ASB 3-3 might have the ability to adhere to suspended articles in the surrounding environment, indicating that it could adhere to organic/inorganic particles available in the Arctic polar environment when its secreted by strain ASB 3-3.

The flocculating stability of EPS at different temperatures was determined and results show that flocculating activity of Arctic EPS is relatively thermally stable (Table 2). The maximum flocculating activity (91.99 ± 1.48%) was observed at 37 °C and it still retained 76.03 ± 1.09% flocculating activity after treatment at 60 °C due to its structure which is mainly composed of polysaccharide. Literature has evidenced that the EPS produced by different microorganisms has high flocculating activity after being heated 60–120 °C and the bioflocculant (EPS) could retain its activity due to its polysaccharide backbone.[4,14,32] The polysaccharide nature of produced polymer from strain ASB 3-3 is consistent with its thermal stability because polysaccharide-natured bioflocculants (EPS) are typically resistant to relatively high temperature. In this study, the average flocculating activity of this EPS was more than 60% during 15 to 60 °C treatment.

The effect of the pH on EPS flocculating activity is shown in Table 2. The EPS is tolerant to extreme pH and showed activity either in a strongly acidic solution (pH 5) or in strongly basic solution (pH above 8). More than 70% removal rate was observed for kaolinite suspension at basic pH range (8–11). The maximum activity was achieved at pH 8 with 82.76 ± 1.38% and a slight decrease in flocculating activity was recorded at pH 12. Flocculating activity of our purified EPS was slightly higher in basic than acidic solution. The pH of the solution plays an important role in flocculating activity of EPS and pH could affect the stability of the suspended particles and the formation of flocs.[6,36] The gradual decline of flocculation activity of EPS with increasing pH possibly results from high alkaline degradation effects on polysaccharide, causing molecular rearrangement of the residues or fragmentation of the polysaccharide chain.[12] The EPS produced from the strain ASB 3-3 possesses a wide range of pH stability (5.0–12.0) and it was highly comparable with existing literatures.[12,32] The high temperature and pH stability characteristics of EPS of ASB 3-3 are favourable for its use under extreme conditions for wastewater treatment industrial applications. According to the present findings and previous reports, the emulsification and flocculation ability of Arctic bacterial EPSs are essential for the formation of aggregates, adhesion to ice surfaces, biofilm formation and sequestering of nutrients, and thus also provide protection and ecological balance in the polar environments.[15,17,18]

3.4.3. Cryoprotective activity of the EPS. The cryoprotective activity of the Arctic bacterial EPS on the survival of both polar strain ASB 3-3 and *E. coli* DH5α cells after multiple freeze–thaw cycles are shown in Fig. 6. With an increase in the EPS concentration from 0 to 50 mg mL^{-1}, the number of surviving cells of both strain ASB 3-3 and *E. coli* DH5α increased after 2nd, 4th, 6th and 8th freeze–thaw cycles, indicative of that the purified Arctic bacterial EPS had a cryoprotective consequence on both strain ASB 3-3 and *E. coli* DH5α. The 0 cycle experiment was also performed to compare the bacterial count after repeating freeze–thaw cycles. In the presence of 50 mg mL^{-1} EPS, the number of surviving cells of strain ASB 3-3 after 2nd freeze–thaw cycles was 38.33 ± 2.05 CFU × 10^{-5} mL^{-1}, which was four times higher than the control (Fig. 6A). The number of surviving cells of *E. coli* DH5α after 2nd freeze–thaw cycles was 19.33 ± 2.86 CFU × 10^{-5} mL^{-1}, which was also four times higher than the control (Fig. 6B). However the presence of the purified EPS could increase the survival of strain ASB 3-3 and *E. coli* DH5α

Table 2 Effect of EPS concentration, temperature and pH on flocculation activity of EPS

EPS concentration (mg L^{-1})	Flocculating activity (%)	pH	Flocculating activity (%)	Temperature (°C)	Flocculating activity (%)
5	49.45 ± 9.12	5	18.89 ± 0.84	4	12.11 ± 1.60
10	64.19 ± 0.60	6	34.99 ± 2.26	15	61.20 ± 1.48
15	66.44 ± 2.27	7	78.90 ± 2.25	25[a]	79.02 ± 2.03
20[a]	70.93 ± 1.75	8[a]	82.76 ± 1.38	30	83.06 ± 4.45
25	73.02 ± 1.12	9	73.04 ± 1.57	37	91.99 ± 1.48
30	74.24 ± 4.84	10	70.24 ± 0.58	40	85.40 ± 2.07
35	84.95 ± 1.22	11	70.27 ± 7.24	50	79.94 ± 0.61
40	91.27 ± 1.01	12	67.07 ± 2.62	60	76.03 ± 1.09

[a] Constant invariables: EPS, 20 mg L^{-1}; pH, 8.0; temperature, 25 °C. The values shown are means ± SDs from three experimental replicates.

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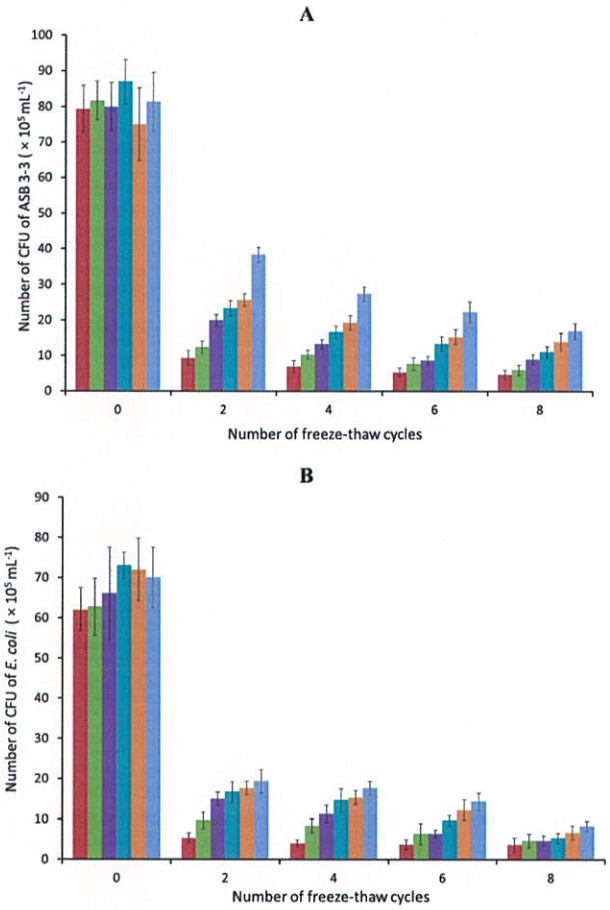

Fig. 6 Cryoprotective activity of the EPS on Arctic *Flavobacterium* sp. strain ASB 3-3 (A) and *E. coli* DH5α (B) after 0, 2, 4, 6 and 8th freeze–thaw cycles (−74 °C). The values shown are means ± SDs from three experimental replicates. Maroon bars, absence of EPS; light green bars, 5.0 mg mL^{-1} EPS; purple bars, 10 mg mL^{-1} EPS; dark cyan bars, 20 mg mL^{-1} EPS; light orange bars, 30 mg mL^{-1} EPS; navy blue bars, 50 mg mL^{-1} EPS.

after recurrent freeze–thaw cycles, the number of surviving cells of both strain ASB 3-3 and *E. coli* DH5α was reduced with an increase in the number of freeze–thaw cycles in the presence/or absence of the purified EPS. In the presence of 50 mg mL^{-1} purified EPS, the number of surviving cells of strain ASB 3-3 was reduced from 38.33 ± 2.05 to 27.33 ± 2.05, 22.33 ± 2.86 and 17 ± 2.16 CFU × 10^{-5} mL^{-1} after 4th, 6th and 8th freeze–thaw cycles, respectively, and that of *E. coli* DH5α was reduced from 19.33 ± 2.86 to 17.66 ± 1.69, 14.33 ± 2.05 and 8.33 ± 1.24 CFU × 10^{-5} mL^{-1}, respectively. The ecological roles of EPSs from bacteria are linked to their ecological niches and their natural environment from which they have been isolated.[16] Freeze–thaw cycles are rather common in the polar regions of the Arctic and Antarctic environment and there are very few reports are available in the aspect of cryoprotective effect of EPS isolated from polar bacteria.[3,20] There has been no report on the cryoprotective activity of EPSs from Arctic soil bacteria. Our result showed that the EPS produced by the Arctic sea strain ASB 3-3 significantly improved the survival of the strain during recurring freeze–thaw cycles, which suggests that the EPS produced by strain ASB 3-3 would be beneficial for the strain to adapt to the freeze–thaw polar environment. In addition, the EPS also promotes the survival rate of *E. coli*, which suggests that, this EPS might have biotechnological potential as a cryoprotective agent.

4. Conclusions

This work describes a novel EPS produced from a psychrotrophic Arctic soil bacterium *Flavobacterium* sp. ASB 3-3 grown on glycerol. The produced EPS polymer is a highly complex heteropolysaccharide composed of repeating units of glucose and galactose. The strain ASB 3-3 produces a high quantity of EPS and the EPS showed an excellent emulsifying and flocculating activity, suggesting its potential industrial utility in enhanced oil recovery or bioremediation of hydrocarbons and waste water treatment. This EPS also exhibits a significant cryoprotective effect on both *Flavobacterium* sp. ASB 3-3 and *E. coli* DH5α, and it can be used as a microbial cryoprotective agent.

Acknowledgements

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References

1 F. Freitas, V. D. Alves, J. Pais, N. Costa, C. Oliveira, L. Mafra, L. Hilliou, R. Oliveira and M. A. M. Reis, *Bioresour. Technol.*, 2009, **100**, 859–865.
2 I. Llamas, H. Amjres, J. A. Mata, E. Quesada and V. Béjar, *Molecules*, 2012, **17**, 7103.
3 A. Poli, G. Anzelmo and B. Nicolaus, *Mar. Drugs*, 2010, **8**, 1779.
4 G. Sathiyanarayanan, V. Vignesh, G. Saibaba, A. Vinothkanna, K. Dineshkumar, M. B. Viswanathan and J. Selvin, *RSC Adv.*, 2014, **4**, 22817–22827.
5 M. M. Camargo-de-Morais, S. A. F. Ramos, M. C. B. Pimentel, M. A. de Morais Jr and J. L. Lima Filho, *World J. Microbiol. Biotechnol.*, 2003, **19**, 191–194.
6 L. Wang, F. Ma, Y. Qu, D. Sun, A. Li, J. Guo and B. Yu, *World J. Microbiol. Biotechnol.*, 2011, **27**, 2559–2565.
7 U. J. Yun and H. D. Park, *Lett. Appl. Microbiol.*, 2003, **36**, 282–287.
8 P. L. Pham, I. Dupont, D. Roy, G. Lapointe and J. Cerning, *Appl. Environ. Microbiol.*, 2000, **66**, 2302–2310.

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9 R. Tallon, P. Bressollier and M. C. Urdaci, *Res. Microbiol.*, 2003, **154**, 705–712.
10 T. Gutierrez, T. Shimmield, C. Haidon, K. Black and D. H. Green, *Appl. Environ. Microbiol.*, 2008, **74**, 4867–4876.
11 A. Dhasayan, J. Selvin and S. Kiran, *3 Biotech*, 2015, **5**, 443–12.
12 W. Tang, L. Song, D. Li, J. Qiao, T. Zhao and H. Zhao, *PLoS One*, 2014, **9**, e114591.
13 S. Deng, R. Bai, X. Hu and Q. Luo, *Appl. Microbiol. Biotechnol.*, 2003, **60**, 588–593.
14 K. Okaiyeto, U. U. Nwodo, L. V. Mabinya and A. I. Okoh, *Int. J. Environ. Res. Public Health*, 2013, **10**, 5097–5110.
15 C. A. M. Nichols, J. Guezennec and J. P. Bowman, *Mar. Biotechnol.*, 2005, **7**, 253–271.
16 S.-B. Liu, X.-L. Chen, H.-L. He, X.-Y. Zhang, B.-B. Xie, Y. Yu, B. Chen, B.-C. Zhou and Y.-Z. Zhang, *Appl. Environ. Microbiol.*, 2013, **79**, 224–230.
17 C. A. Mancuso Nichols, S. Garon, J. P. Bowman, G. Raguénès and J. Guézennec, *J. Appl. Microbiol.*, 2004, **96**, 1057–1066.
18 C. Nichols, S. Lardière, J. Bowman, P. Nichols, J. A. E. Gibson and J. Guézennec, *Microb. Ecol.*, 2005, **49**, 578–589.
19 G. Qin, L. Zhu, X. Chen, P. G. Wang and Y. Zhang, *Microbiology*, 2007, **153**, 1566–1572.
20 L. Selbmann, S. Onofri, M. Fenice, F. Federici and M. Petruccioli, *Res. Microbiol.*, 2002, **153**, 585–592.
21 D. H. Bergey and R. S. Breed, *Bergey's manual of determinative bacteriology*, Williams & Wilkins Co., Baltimore, 1957.
22 G. Sathiyanarayanan, G. Saibaba, G. Seghal Kiran and J. Selvin, *Bioprocess Biosyst. Eng.*, 2013, **36**, 1817–1827.
23 V. Vignesh, G. Sathiyanarayanan, G. Sathishkumar, K. Parthiban, K. S. Kumar and R. Thirumurugan, *RSC Adv.*, 2015, **5**, 27794–27804.
24 M. DuBois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, 1956, **28**, 350–356.
25 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248–254.
26 T. T. Terho and K. Hartiala, *Anal. Biochem.*, 1971, **41**, 471–476.
27 J. P. Bowman, S. A. McCammon, M. V. Brown, D. S. Nichols and T. A. McMeekin, *Appl. Environ. Microbiol.*, 1997, **63**, 3068–3078.
28 C. E. Starliper, *J. Adv. Res.*, 2011, **2**, 97–108.
29 J. Farkas, *Aquaculture*, 1985, **44**, 1–10.
30 E. Hantsis-Zacharov and M. Halpern, *Appl. Environ. Microbiol.*, 2007, **73**, 7162–7168.
31 H. Daskalov, D. A. Austin and B. Austin, *Lett. Appl. Microbiol.*, 1999, **28**, 297–299.
32 M. Nie, X. Yin, J. Jia, Y. Wang, S. Liu, Q. Shen, P. Li and Z. Wang, *J. Appl. Microbiol.*, 2011, **111**, 547–558.
33 N. Gorret, J. L. Maubois, J. M. Engasser and M. Ghoul, *J. Appl. Microbiol.*, 2001, **90**, 788–796.
34 F. de Alexandre Sebastião, F. Pilarski and M. V. F. Lemos, *Braz. J. Microbiol.*, 2013, **44**, 861–864.
35 Z. Zulkeflee, A. Z. Aris, Z. H. Shamsuddin and M. K. Yusoff, *Sci. World J.*, 2012, **2012**, 495659.
36 H. Yokoi, O. Natsuda, J. Hirose, S. Hayashi and Y. Takasaki, *J. Ferment. Bioeng.*, 1995, **79**, 378–380.

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