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

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Ganesan Sathiyarayanan<sup>a</sup>, Da-Hye Yi<sup>a</sup>, Shashi Kant Bhatia<sup>a</sup>, Jung-Ho Kim<sup>a</sup>, Hyung Min Seo<sup>a</sup>, Yun-Gon Kim<sup>b</sup>, Sung-Hee Park<sup>c</sup>, Daham Jeong<sup>d,e</sup>, Seunho Jung<sup>d,e</sup>, Ji-Young Jung<sup>f</sup>, Yoo Kyung Lee<sup>d</sup>, Yung-Hun Yang<sup>a,e,\*</sup>

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) was obtained after cultivation at 25 °C for 120 h with glycerol as sole carbon source. The EPS was purified and its structural characteristics were analyzed by <sup>1</sup>H and <sup>13</sup>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 has mannose. In addition, EPS has demonstrated comparable emulsifying property to 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 region and it can have possible usage as microbial cryoprotectants.

### 1. Introduction

The increased demand of natural polysaccharides for industrial applications has led to increased consideration in the 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 the presence of excess of carbon source in the growth medium or under stressful environment.<sup>1-4</sup> 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.<sup>5-7</sup> The EPSs are also used in medical field as anti-tumor, anti-viral, and anti-inflammatory agents.<sup>8,9</sup> At present, there has been an increasing interest in search of novel EPSs and few of them are being currently marketed as commercial products include

xanthan, alginates, cellulose, pullulan, gellan, hyaluronic acid and succinoglycan from different bacterial strains.<sup>1,4,5</sup>

Emulsifier and flocculants have been used extensively in almost every industrial sector of modern industry today.<sup>10</sup> The large fraction of emulsifier and flocculants are produced by chemical synthesis, 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 an alternative emulsifier and flocculant.<sup>4,5,11</sup> Biopolymers are biodegradable and their intermediates and by-products are harmless towards human being and the environment. In addition, microbial derived polymers can exhibit enhanced performance and greater functional diversity than synthetic polymers.<sup>5,12</sup> It has been reported that many bacteria able to produce EPS based emulsifying and flocculating agents for enhanced oil recovery/ or hydrocarbon degradation and waste water treatment, respectively.<sup>2,3,5,13,14</sup> 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.<sup>7,9</sup> Due to this, the vast numbers of microbial strains are being evaluated to find out the novel EPSs for commercial applications.<sup>4</sup> Currently, Arctic and Antarctic polar bacteria are recognized as a rich source of biological macromolecules that are of potential interest towards various industrial applications<sup>15</sup> and to date, only very few reports are available on EPS from polar bacteria and their industrial prospects.<sup>15-20</sup> In the polar environment, bacterial EPSs are essential for the formation of

<sup>a</sup>Department of Biological Engineering, College of Engineering, Konkuk University, Seoul 143-701, South Korea

<sup>b</sup>Chemical Engineering, Soongsil University, 511 Sangdo-dong, Seoul 156-743, South Korea,

<sup>c</sup>Food Ingredients Center, Foods R&D, CheilJedang, Guro-dong, Guro-Gu, Seoul 152-051, South Korea,

<sup>d</sup>Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, South Korea

<sup>e</sup>Microbial Carbohydrate Resource Bank, Konkuk University, Seoul 143-701, South Korea

<sup>f</sup>Division of Life Sciences, Korea Polar Research Institute, 12 Gaetbeol-ro, Yeosu-si, Incheon 406-840, South Korea

\*Corresponding author. Tel.: +82-2-450-3936; Fax: +82-2-3437-8360. E-mail address: seokor@konkuk.ac.kr (Y.-H. Yang)

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aggregates, adhesion to or colonization of surfaces, formation of biofilms and possibly acting as ligands for trace metals sequestration (nutrients) or providing cryoprotection for the growth at low temperature and high salinity.<sup>3, 15, 18</sup> 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.<sup>3, 15, 18</sup> 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 near by moving glaciers and followed by the production and structural characterization of a novel EPS from psychrotrophic Arctic strain *Flavobacterium* sp. ASB 3-3 are described. 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 sections

### 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, Norway 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  $-80\text{ }^{\circ}\text{C}$  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  $20\text{ }^{\circ}\text{C}$  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 were incubated at 4, 15, 20 and  $25\text{ }^{\circ}\text{C}$  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<sup>4</sup> and the composition of screening medium included sucrose, 20 g/L;  $\text{NH}_4\text{NO}_3$ , 1.0 g/L; yeast extract, 0.1 g/L;  $\text{KH}_2\text{PO}_4$ , 0.3 g/L;  $\text{K}_2\text{HPO}_4$ , 0.3 g/L;  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ , 0.1 g/L;  $\text{MnSO}_4\cdot 4\text{H}_2\text{O}$ , 0.1 g/L;  $\text{CaCO}_3$ , 0.4 g/L; NaCl, 0.05 g/L and tryptone, 0.1 g/L with the initial pH  $7.0 \pm 0.2$ . 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  $25\text{ }^{\circ}\text{C}$ . 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 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 Bergey's Manual of Determinative Bacteriology.<sup>21</sup> 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').<sup>22</sup> A 25  $\mu\text{L}$  reaction volume PCR was performed using about 10 ng of genomic DNA, 1X reaction buffer (10 mM Tris-HCl pH 8.8, 1.5 mM  $\text{MgCl}_2$ , 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  $95\text{ }^{\circ}\text{C}$  for 3 min, then 30 cycles consisting of  $95\text{ }^{\circ}\text{C}$  for 45 sec,  $55\text{ }^{\circ}\text{C}$  for 45 sec,  $72\text{ }^{\circ}\text{C}$  for 1.45 min and finally an extension step at  $72\text{ }^{\circ}\text{C}$  for 10 min. The PCR amplicon was cloned by the TA cloning method using TOPO TA Cloning kit according to manufactures 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://blast.ncbi.nlm.nih.gov/Blast.cgi>) for their pair wise 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 6.0 version ([www.megasoftware.net](http://www.megasoftware.net)) 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 ASB3-3

The Arctic strain ASB 3-3 was inoculated into culture medium containing glucose, 10 g/L; yeast extract, 0.5 g/L;  $\text{KH}_2\text{PO}_4$ , 0.2 g/L; NaCl, 0.1 g/L;  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ , 0.1 g/L 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 medium was as follows: Glycerol, 30 g/L; yeast extract, 0.5 g/L;  $\text{NH}_4\text{H}_2\text{PO}_4$ , 3.3 g/L;  $\text{KH}_2\text{PO}_4$ , 3.7 g/L;  $\text{K}_2\text{HPO}_4$ , 5.8 g/L;  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  (100 mM), 10 mL/L and micronutrient solution (MNS), 1 mL/L. The MNS comprises  $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ , 2.18 g/L;  $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ , 1.98 g/L;  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ , 1.67 g/L;  $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$ , 0.17 g/L and  $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ , 0.29 g/L. The initial pH of the medium was adjusted to  $7.0 \pm 0.2$ . The inoculated flasks were incubated with 150 rpm agitation at  $25\text{ }^{\circ}\text{C}$  for 7 days. All experiments were performed in triplicates. After incubation, the culture volume of 1 L was pooled and centrifuged at  $12,000 \times g$  for 30 min at  $4\text{ }^{\circ}\text{C}$  and the cell pellet (Biomass) was lyophilized and weighed. The EPS extraction was performed according to literatures.<sup>4, 23</sup> The cell-free supernatant (CFS) was subjected to thermal treatment ( $80\text{ }^{\circ}\text{C}$ , 1h) 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 volume of ice cold absolute ethanol was added into concentrated CFS in a 500 mL of Erlenmeyer flasks and kept at  $4\text{ }^{\circ}\text{C}$  for overnight for the EPS precipitation. The exopolymeric precipitate was collected by high speed centrifugation at  $14,000 \times g$  for 30 min

at 4 °C. The precipitate was washed with 70–100% ethanol-water mixtures. After ethanol-water washing for thrice, the precipitates which were pooled and dried in a desiccator and stored at room temperature till needed. The EPS precipitate was dissolved in deionized water and its proteins contents (impurities) were removed (deproteinization) by trichloroacetic acid and Savag method.<sup>16</sup> The deproteinization was repeated twice and the extracted EPS were combined and re-dissolved 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 lyophilized. The purified EPS was stored at room temperature until physical and chemical analyses 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<sup>-1</sup>) and the total carbohydrate contents were assayed by phenol sulphuric acid method with glucose as standard.<sup>24</sup> The total protein content was determined with bovine serum albumin (BSA) as standard using Bradford assay.<sup>25</sup> Sulfated sugars were calculated by measuring the amount of sulfate content using K<sub>2</sub>SO<sub>4</sub> as standard.<sup>26</sup> 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 5mM sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) as eluent, at a flow rate of 0.600 mL/min with oven temperature of 50 °C.

### 2.4.3. Fourier Transform Infrared (FT-IR) Spectrometer

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<sup>-1</sup> in 4000-600 cm<sup>-1</sup> region. Spectra were corrected for wave number-dependent signal detection efficiency of the setup using the white light spectrum of a temperature-calibrated tungsten band lamp.

### 2.4.4. <sup>13</sup>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 at temperature range -50 °C to 80 °C. The purified EPS of the strain ASB 3-3 was dissolved in D<sub>2</sub>O at concentrations of 5 mg mL<sup>-1</sup> (for <sup>1</sup>H NMR) and 20 mg mL<sup>-1</sup> (for <sup>13</sup>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<sub>24</sub>]) was measured after 24 h incubation at 25 °C. The emulsification index (EI<sub>24</sub>) was calculated using a formula:

$$\text{Emulsification index (EI}_{24}) = \frac{\text{HEL}}{\text{HS}} \times 100\% \quad (1)$$

Where, HEL is the height of the emulsified layer and HS is the height of the total liquid column. Sterile distilled water was used as the negative control.<sup>11</sup> This same assay was also used to measure the EI<sub>24</sub> produced by the solutions of the extracted EPS against hydrocarbons such as n-hexadecane (99%, Alfa Aesar), methyl octonate (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<sub>2</sub>Si<sub>2</sub>O<sub>5</sub>(OH)<sub>4</sub>) as the suspended solid. Briefly, 5.0 mL of 1% (w/v) CaCl<sub>2</sub> and 0.2 mL of EPS of strain ASB 3-3 (5 mg L<sup>-1</sup>) were added into 95 mL of Kaolinite suspension (5.0 g/L, 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 EPS and the 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<sup>-1</sup>. The pH of the Kaolinite suspension was adjusted using 1M NaOH and 1N 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<sub>600</sub>= 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<sup>-1</sup>) 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 1h 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, 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup> or 8<sup>th</sup> thawing, the mixture was properly

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 in thrice. Data were expressed as mean  $\pm$  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 3-3

Total 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 exhibited the significant EPS productivity than other reference strains. The EPS yields of those strains under our experimental conditions were in the range of  $0.41 \pm 0.09$  to  $6.24 \pm 0.73$  g/L (Fig. 1). Strain ASB 3-3 grew well in both sucrose and glycerol enriched medium, which exhibits their maximum EPS productivity ( $6.24 \pm 0.73$  g/L) 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 showed the moderate productivity, which was lesser than 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://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 revealed that the organism was *Flavobacterium*, in which many Arctic and Antarctic strains are found.<sup>18</sup> Representative of maximum homologous (98–99%) sequences of each isolate were obtained from 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.

Studies on the diversity of Arctic and Antarctic polar microbial communities were 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.<sup>18, 27</sup> *Flavobacterium*, a genus of the Gammaproteobacteria under *Flavobacteriaceae* family and it's

a universal commensal bacteria that live in soil, water and few of them are opportunistic pathogens.<sup>28</sup> 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.<sup>18</sup> The strain ASB 3-3 isolated from Ny-Alesund, Midtre Lovénbreen, exhibited the psychrotrophic growth pattern and our results corroborates with previous studies about the existence of psychrotrophic nature of *Flavobacterium* in natural environments.<sup>28-30</sup> 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 5 day's incubation in liquid media at 25 °C, robust growth was observed and then it was termed as 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 ( $OD_{595\text{nm}}$ ,  $3.86 \pm 0.17$ ) 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 120h of incubation. The maximum synthesis of EPS was reached at early stationary phase of growth and it was slightly decreased after 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  $7.2 \pm 0.02$  to  $5.6 \pm 0.15$  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 about  $7.25 \pm 0.26$  g/L was achieved with biomass of  $3.18 \pm 0.27$  ( $OD_{595\text{nm}}$ ) from the Arctic strain ASB 3-3. The EPS production in bacteria isolated from cold environment including Arctic and Antarctic region was compared (Table 1) and the strain ASB 3-3 producing high amount of EPS among the polar strains.

The *Flavobacterium* is a slow growing bacterium<sup>31</sup> and it was also observed the same from 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 the nutrient starvation or cell autolysis.<sup>14</sup> The decrease on EPS productivity observed after 120 h could be attributed to the accumulation of EPS-degrading enzymes produced by the bacterium.<sup>32</sup> A similar observation was also reported with other microorganisms such as EPS-producing lactic-acid bacteria and halophilic bacterium *Halomonas almeriensis*,<sup>8, 33</sup> since EPS yields increased during the log phase and stopped when the stationary growth phase was reached.<sup>2</sup> These results do not agree with those of other authors, however, who maintain that

cell growth and EPS formation usually have different nutritional requirements.<sup>9, 13</sup> 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.<sup>14, 32</sup>

### 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 the both carbohydrate and protein parts. After the deproteinization (Sevag method) and desalting (dialysis) processes completely removed the proteins and salts, respectively, which results the 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 sulphuric acid method was periodically performed along with HPLC analysis (data not shown), which provides an enough evidence for the purity of EPS.

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,<sup>18, 34</sup> 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 a 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.<sup>1, 4</sup> 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.<sup>1</sup>

#### 3.3.2. Functional group analysis by FT-IR spectroscopy

The FT-IR spectrum of purified EPS showed (Fig. 4A) diverse range of absorption peaks from 3450 to 600  $\text{cm}^{-1}$ . Presence of high levels of hydroxyl groups (O-H) stretching frequencies was identified by broad absorption peak around 3000–3500  $\text{cm}^{-1}$  confirming the polysaccharide nature of the purified exopolymer. Two weak absorption peaks at 2921 and 2815  $\text{cm}^{-1}$  were assigned to C-H asymmetric stretch and C-H symmetric stretch of  $\text{CH}_2$  and  $\text{CH}_3$  groups, respectively. The strong absorption band at 1585 and small peak at 1618  $\text{cm}^{-1}$  were

indicates the presence of carbonyl (C=O) and amide or amine (C=O, C-N) group, respectively in the EPS material. A strong symmetrical stretching band at 1308  $\text{cm}^{-1}$  showed the presence of carboxyl (C=O) of -COO groups. The absorption peak at 1076  $\text{cm}^{-1}$  corresponds to the saccharide moieties and strong absorption around 600–950  $\text{cm}^{-1}$  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), hydroxyl (-OH), amide (C=O, C-N) and carboxyl (-COO), which may act as a receptor for divalent cations ( $\text{Ca}^{2+}$ ) during flocculating activity.<sup>4</sup> Two main roles in flocculation are important characteristics of these functional groups: hydrophobicity characteristics are utilized to extend the polymer chain; and the functional groups extend the gap between the objects to adsorb the organic particles.<sup>12</sup> A stretching band around 1600  $\text{cm}^{-1}$  in the EPS of *Pseudomonas oleovorans* NRRL B-14682, which can be attributed to ring structure of galactose and other sugar moieties<sup>1</sup>, which is also observed in the EPS of ASB 3-3.

#### 3.3.3. Structure analysis by solid state NMR

The  $^1\text{H}$  proton NMR spectrum showed two anomeric resonance signals at  $\delta$  4.9 and 5.2 ppm are corresponds to proton of the first carbon of the monosaccharide's C-1 (Fig. S4) and a number of overlapping signals at  $\delta$  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  $\delta$  3.8 and 3.81 ppm should belong to C-6 (H) of the glucose and galactose and these signals may due to reduced end-chains. The signals at  $\delta$  4.91–4.20 ppm shifts were characteristic of glucose and galactose moieties.  $^1\text{H}$  proton NMR spectrum appears as singlets but indicating that the same monosaccharide at different locations resulted in anomeric proton shift. Since, it is both glucose and galactose are epimers. Due to this reason, for an EPS in  $\text{D}_2\text{O}$  all glucose proton signals merged with other signal owing to a fast exchange in NMR time scale.

$^{13}\text{C}$  NMR resonances of the EPS are much more dispersed than their  $^1\text{H}$  counterparts (Fig. 4B). The two main signals in the anomeric region at  $\delta$  103 (doublets as 103.50 and 103.57) and 98.77 (singlet) ppm and were assigned to C-1 position of  $\alpha$  or  $\beta$ -D-galactose and  $\beta$ -D-glucose residues, respectively. There is one more singlet at  $\delta$  94.97 ppm also observed and it may belongs to anomeric C-1 position of  $\alpha$ -D-glucose. A small resonance at  $\delta$  100.55 ppm was assigned to (1 $\rightarrow$ 6)-linked  $\alpha$ -D-glucose residues. The chemical shifts around  $\delta$  70–78 ppm were assigned to C-2, C-3, C-4 and C-5 of glucose ( $\alpha$  or  $\beta$ ). The main peaks observed at  $\delta$  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 ( $\alpha$  or  $\beta$ ) hydroxyl substitution of galactose. In addition, the observed  $\delta$  values for the C-6 of the glucose ( $\alpha$  or  $\beta$ ) and galactose ( $\alpha$  or  $\beta$ ) were  $\delta$  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  $\alpha$  and  $\beta$ -D-glucopyranose rings connected with (1 $\rightarrow$ 6)-glycosidic linkage. From both  $^1\text{H}$  and  $^{13}\text{C}$  NMR, there is no unwanted noise; hence this EPS polymer is 100% pure without any impurities. The structure of this EPS is different from that of EPSs produced by other Arctic/Antarctic bacteria.<sup>3, 18</sup> Previous reports on polar environmental bacteria showed that the mannose is a main component of many EPSs.<sup>15, 17, 18</sup> The EPS produced by *Pseudoalteromonas* spp. and *Flavobacterium* spp. isolated from polar region, has a branched mannan structure comprised of a backbone of mannose residues.<sup>16, 18</sup> In addition, many EPSs

secreted by polar bacteria are polyanionic nature due the presence of uronic acids, ketal-linked pyruvate, and inorganic residues.<sup>3</sup> 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 environment is needed to elucidate the structure of the polymers and to know about 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, at 0.1 to 0.5% did not have any effect on the emulsification of hydrocarbon (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 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 and followed by *n*-hexadecane, with an emulsifying index of 66.33±2.05 and 64.33±3.29%, respectively and it is comparable to the values observed for SDS (63.33±2.49 & 65.96±1.23%). The purified EPS showed less emulsifying activity against with methyl octanoate about 26.33±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±14.36% and based on the average results for all hydrocarbons and EPS produced emulsifying activities that were adequate and considerable from that of the control (SDS). Interestingly, even very low concentration (1%) of EPS has produced stable and 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 products emulsions with the various aromatic hydrocarbons. Further, the stability and high percentage of emulsions is an advantageous property both in terms of bioprocess 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 amphipathic in nature.<sup>5, 10</sup> Carboxylate and methoxycarbonyl groups of polysaccharides have also been reported to contribute emulsifying activities to carbohydrate polymers.<sup>7, 10</sup>

#### 3.4.2 Flocculating activity of the EPS

An optimal concentration of EPS for flocculating activity was determined using kaolinite suspension with constant invariables (EPS, 20 mg L<sup>-1</sup>; pH, 8.0; Temperature, 25 °C). The highest flocculating activity (91.27±1.01%) was observed at the concentration of 40.0 mg L<sup>-1</sup> (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 at the range of 5.0 to 40 mg L<sup>-1</sup> EPS. Usually, low dosage will not make 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.<sup>35</sup> Therefore, 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 with different temperature was determined and results shows 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 evidenced that the EPS produced by different microorganisms has the high flocculating activity after being heated 60–120 °C and the bioflocculant (EPS) could retain its activity due to polysaccharide backbone.<sup>4, 14, 32</sup> 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 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 to 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 suspended particles and the formation of flocs.<sup>6, 36</sup> The gradual decline of flocculation activity of EPS with increasing pH possibly results from high alkaline degradation effects on polysaccharide, causing molecular rearrangement of its residue or fragmentation of the polysaccharide chain.<sup>12</sup> 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.<sup>12, 32</sup> 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.<sup>15, 17, 18</sup>

#### 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<sup>-1</sup>, the number of surviving cells of both strain ASB 3-3 and *E. coli* DH5α increased after 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup> and 8<sup>th</sup> 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<sup>-1</sup> EPS, the number of surviving cells of

strain ASB 3-3 after 2<sup>nd</sup> freeze-thaw cycles was  $38.33 \pm 2.05$  CFU  $\times 10^{-5}$  mL<sup>-1</sup>, which was 4 times higher than the control (Fig. 6A). The number of surviving cells of *E. coli* DH5 $\alpha$  after 2<sup>nd</sup> freeze-thaw cycles was  $19.33 \pm 2.86$  CFU  $\times 10^{-5}$  mL<sup>-1</sup>, which was also 4 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* DH5 $\alpha$  after recurrent freeze-thaw cycles, the number of surviving cells of both strain ASB 3-3 and *E. coli* DH5 $\alpha$  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<sup>-1</sup> purified EPS, the number of surviving cells of strain ASB 3-3 was reduced from  $38.33 \pm 2.05$  to  $27.33 \pm 2.05$ ,  $22.33 \pm 2.86$  and  $17 \pm 2.16$  CFU  $\times 10^{-5}$  mL<sup>-1</sup> after 4<sup>th</sup>, 6<sup>th</sup> and 8<sup>th</sup> freeze-thaw cycles, respectively, and that of *E. coli* DH5 $\alpha$  was reduced from  $19.33 \pm 2.86$  to  $17.66 \pm 1.69$ ,  $14.33 \pm 2.05$  and  $8.33 \pm 1.24$  CFU  $\times 10^{-5}$  mL<sup>-1</sup>, respectively. The ecological roles of EPSs from bacteria are linked to their ecological niches and their natural environment from which they have been isolated.<sup>16</sup> Freeze-thaw cycles are rather common in the polar regions of the Arctic and Antarctic environment and there is very few reports are available in the aspect of cryoprotective effect of EPS isolated from polar bacteria.<sup>3, 20</sup> 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 highly complex heteropolysaccharide composed of repeating units of glucose and galactose. The strain ASB 3-3 produces high quantity of EPS and EPS showed an excellent emulsifying and flocculating activity, suggesting its potential industrial utility in enhanced oil recovery or bioremediation of hydrocarbon and waste water treatment. This EPS also exhibit a significant cryoprotective effect on both *Flavobacterium* sp. ASB 3-3 and *E. coli* DH5 $\alpha$ , and it can be used as microbial cryoprotective agent.

## Acknowledgements

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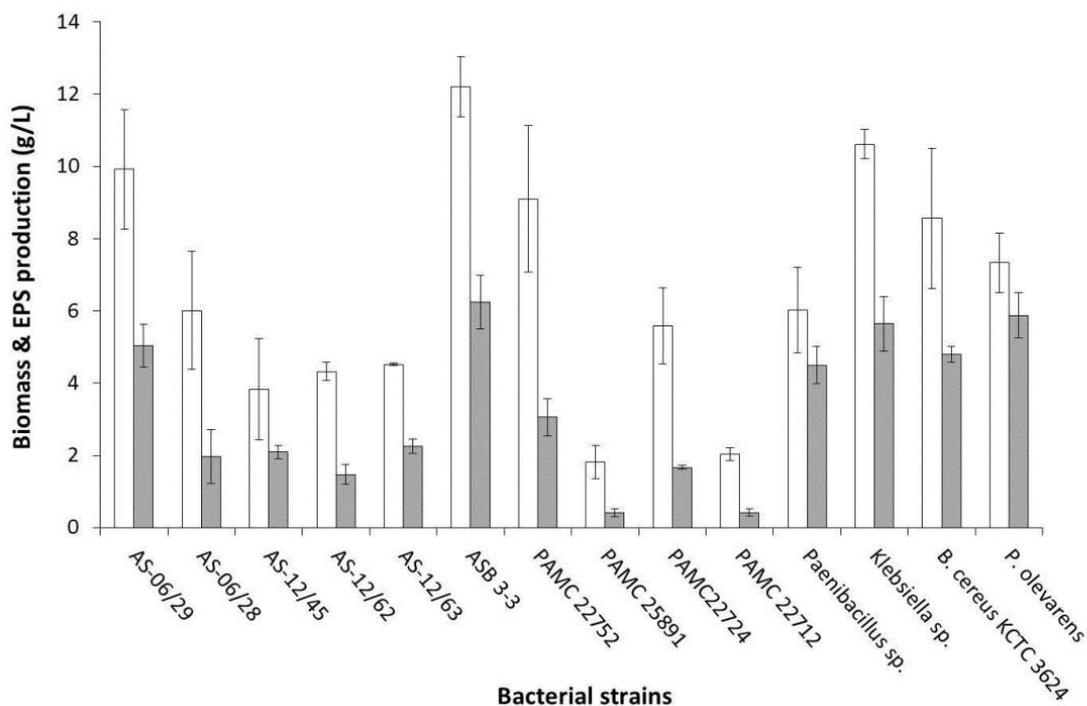
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## ARTICLE

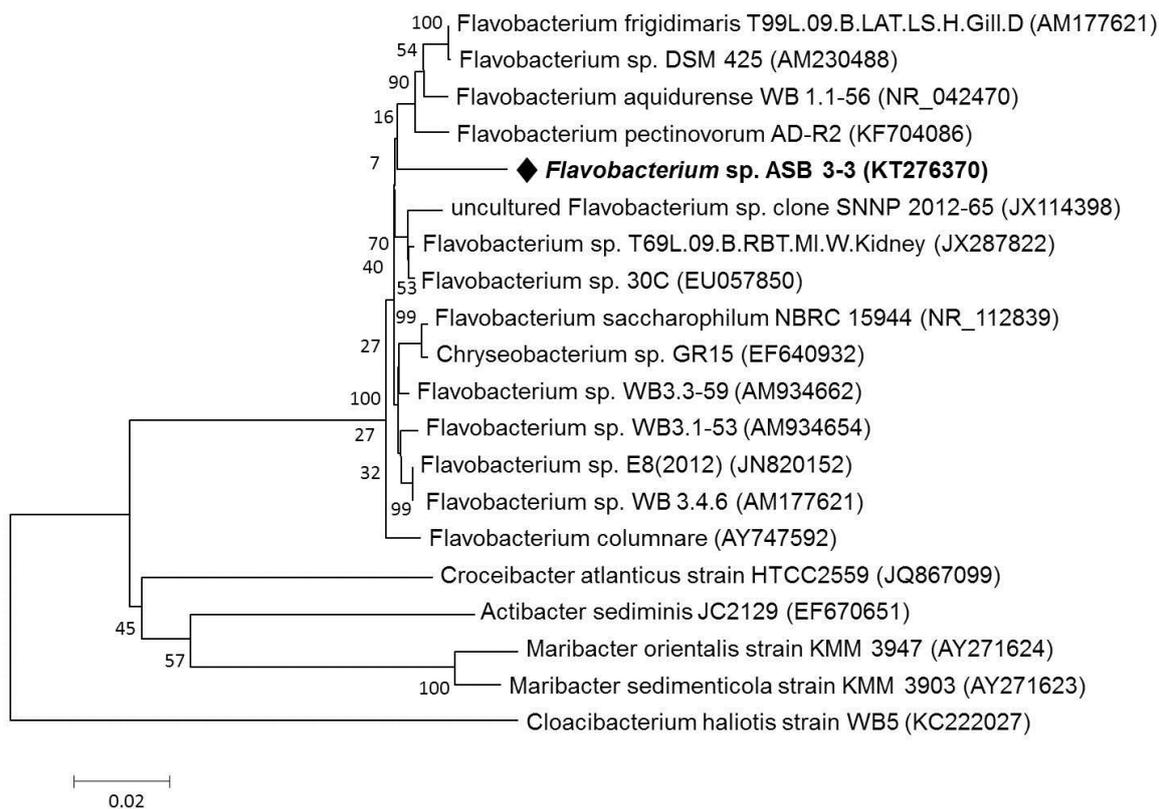
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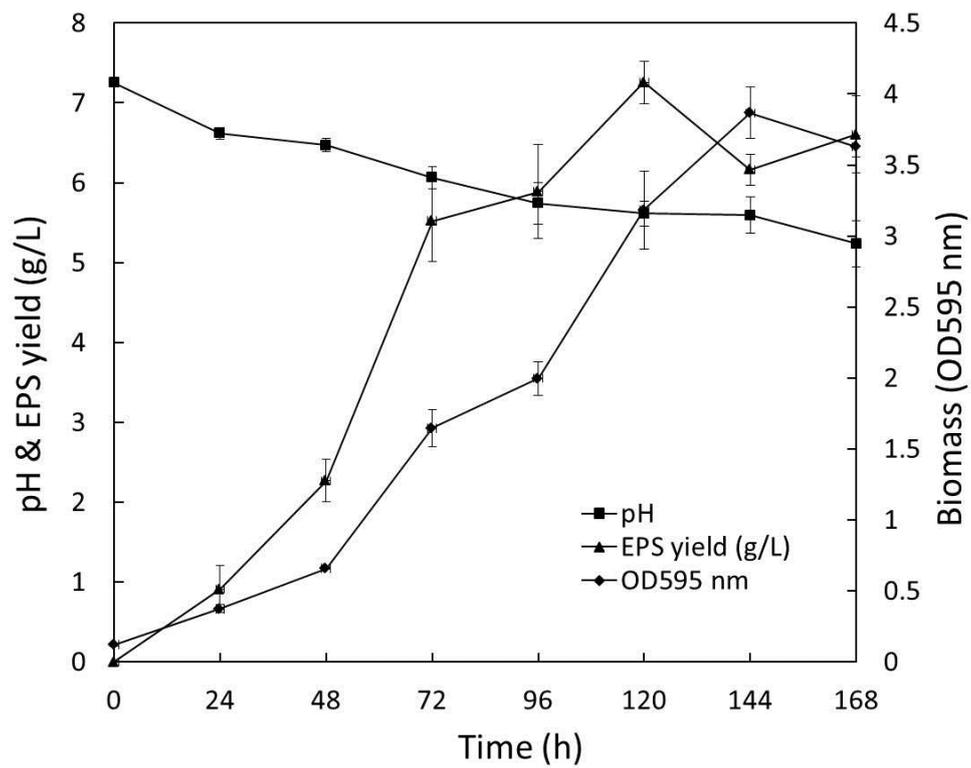
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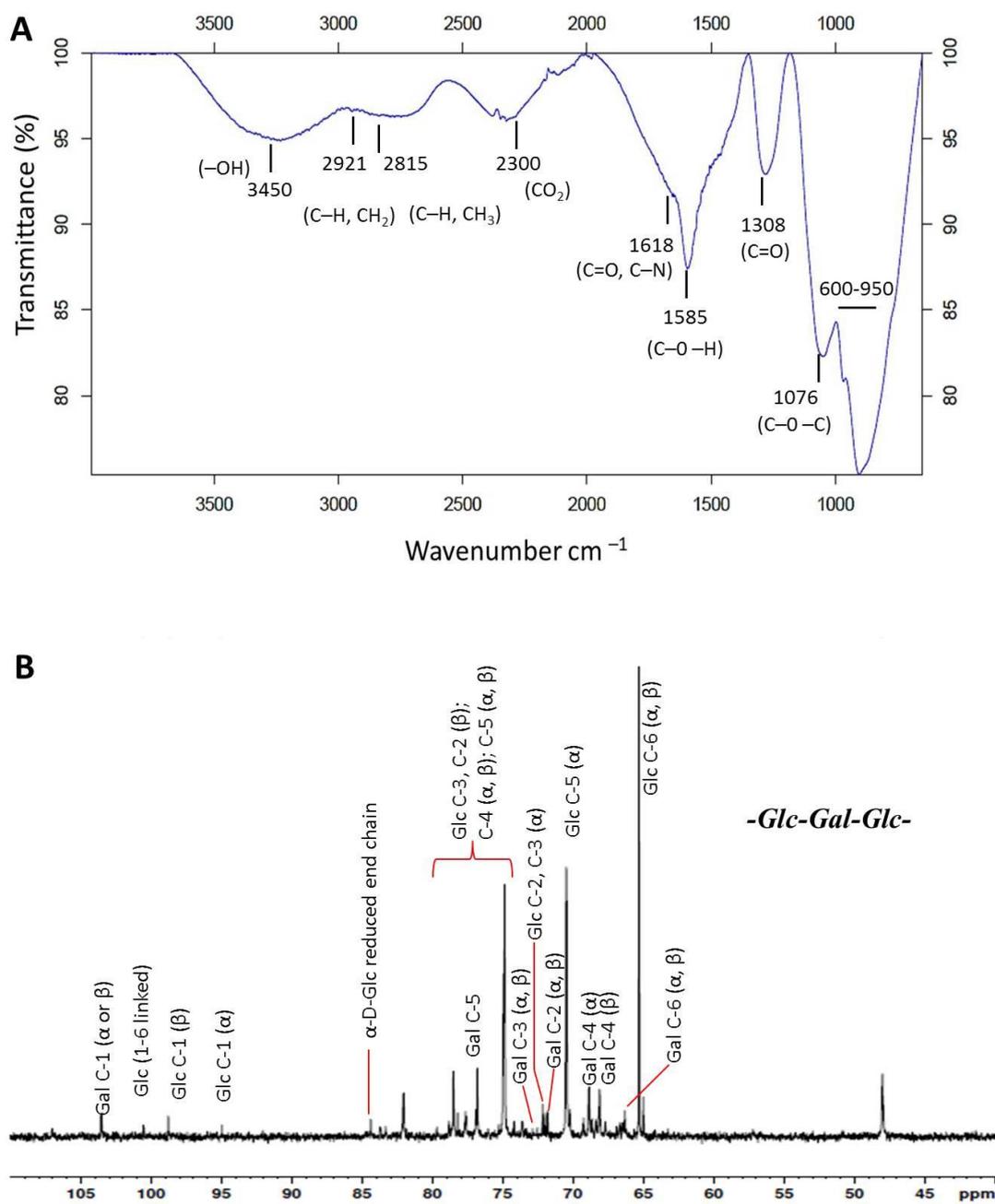
**Fig. 1.** EPS production from 6 Arctic strains (AS & ASB), 4 polar strains (PAMC) and other 4 reference strains in 3% glycerol enriched medium. EPS production was determined by quantifying the whole EPS content after lyophilization. The values shown are means  $\pm$  SDs from three experimental replicates. White color bars, Biomass (g/L); Black color bars, EPS production (g/L)



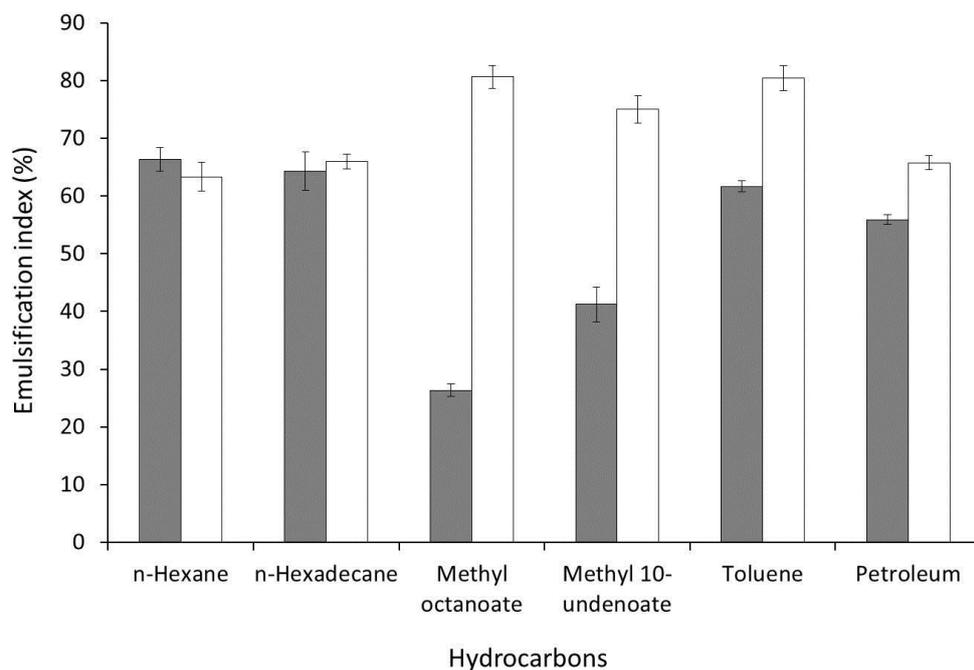
**Fig. 2.** Neighbor-Joining (NJ) bootstrapping (1000) phylogenetic tree of arctic soil bacterium ASB 3-3 and their closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences. Phylogenetic tree was developed based on maximum composite likelihood method using MEGA 6.06 version.



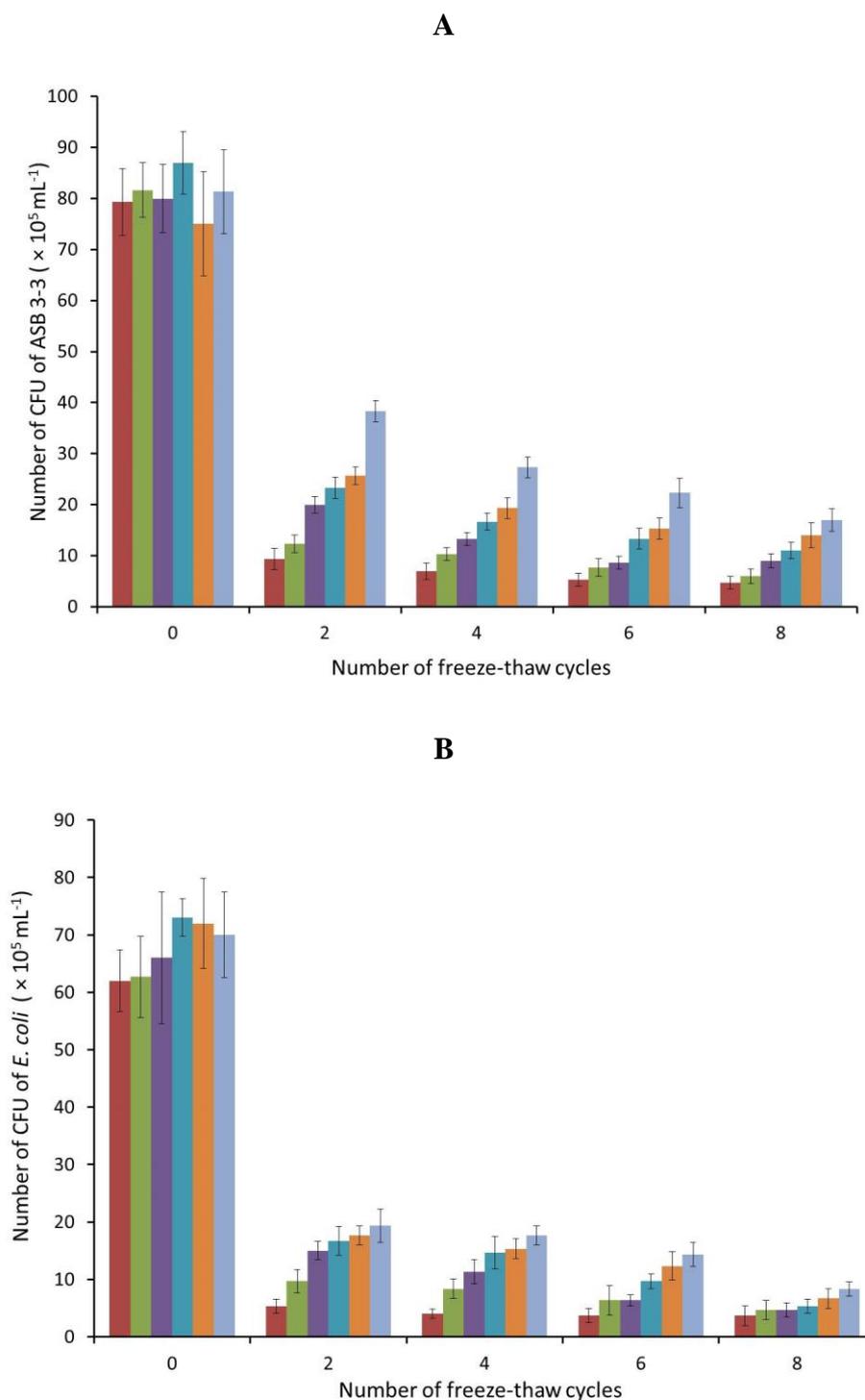
**Fig. 3.** Growth curve of strain ASB 3-3, pH change and EPS production. Strain ASB 3-3 was cultivated in 3% glycerol medium. Error bars indicate the SD.



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



**Fig. 5.** Emulsification activity of the EPS purified from arctic *Flavobacterium* sp. strain ASB 3-3. Emulsification index [EI<sub>24</sub>] after 24 h was calculated with different hydrocarbons. Equal volume sterile water in the place of EPS was used as the control. The values shown are means  $\pm$  SDs from three experimental replicates. Black color bars, 1% EPS from strain ASB 3-3; White color bars, 1% SDS.



**Fig. 6.** Cryoprotective activity of the EPS on arctic *Flavobacterium* sp. strain ASB 3-3 (A) and *E. coli* DH5 $\alpha$  (B) after 0, 2, 4, 6 and 8<sup>th</sup> freeze-thaw cycles ( $-74$  °C). The values shown are means  $\pm$  SDs from three experimental replicates. Maroon bars, absence of EPS; light green bars, 5.0 mg mL<sup>-1</sup> EPS; purple bars, 10 mg mL<sup>-1</sup> EPS; dark cyan bars, 20 mg mL<sup>-1</sup> EPS; light orange bars, 30 mg mL<sup>-1</sup> EPS; navy blue bars, 50 mg mL<sup>-1</sup> EPS.

**Table 1** EPS production in bacteria isolated from cold environment including Arctic and Antarctic region

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

cdw: cell dry weight

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

EPS concentration (mg L <sup>-1</sup> )	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 <sup>a</sup>	79.02±2.03
20 <sup>a</sup>	70.93±1.75	8 <sup>a</sup>	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

<sup>a</sup>Constant invariables: EPS, 20 mg L<sup>-1</sup>; pH, 8.0; temperature, 25 °C. The values shown are means ± SDs from three experimental replicates.

## A table of contents entry

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

Ganesan Sathiyarayanan<sup>a</sup>, Da-Hye Yi<sup>a</sup>, Shashi Kant Bhatia<sup>a</sup>, Jung-Ho Kim<sup>a</sup>, Hyung Min Seo<sup>a</sup>, Yun-Gon Kim<sup>b</sup>, Sung-Hee Park<sup>c</sup>, Daham Jeong<sup>d,e</sup>, Seunho Jung<sup>d,e</sup>, Ji-Young Jung<sup>f</sup>, Yoo Kyung Lee<sup>f</sup>, Yung-Hun Yang<sup>a,e,\*</sup>



Exopolysaccharide from psychrotrophic Arctic glacier soil bacterium *Flavobacterium* sp. ASB 3-3