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Sphingobactan, a new α-mannan exopolysaccharide from Arctic *Sphingobacterium* sp. IITKGP-BTPF3 capable of biological response modification

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ABSTRACT

An exopolysaccharide, from a new Arctic permafrost isolate, *Sphingobacterium* sp. IITKGP-BTPF3 was purified and characterized. Upon optimization of various parameters (pH, temperature, carbon and nitrogen source), the yield of EPS obtained was 1.42 g/L. Structural investigation through FT-IR, GC–MS/MS, HPLC and NMR (1D and 2D) revealed the molecule to be a mannan with α-(1 → 2) and α-(1 → 6) linkages. Anti-oxidant and macrophage immunomodulatory assays were employed for the assessment of bioactivity. Sphingobactan was found to be capable of scavenging superoxide anions, and reducing the nitric oxide production in LPS elicited murine macrophage (RAW 264.7) cell line. The *in vitro* findings indicate the potential of Sphingobactan as a biological response modification (BRM) agent, for containment and possible resolution of inflammatory response *in vivo*.

1. Introduction

Exopolysaccharides (EPS) of bacterial origin, in recent times, are being considered for bio-prospecting by a lot of research groups all over the world. The search for bioactive polysaccharides has ranged from deep sea hydrothermal vents [1] to polar and alpine ice [2] for obtaining novel molecules of interest. Among the four major macromolecules of a living system, namely protein, lipid, nucleic acid and carbohydrate; the last one has received attention in their polymeric form, majorly in terms of industrial production of food, feed and sustainable energy [3]. It is only recently, that the polysaccharides are being investigated and used extensively as biomaterials for various industrial applications [4] and in therapeutic purposes. The advantages that microbial EPS possess over plant or algal ones include novel functionality, chemical and physical properties of reproducible nature and finally their cost effectiveness [5].

Extremophiles are capable of growing in harsh conditions. Cold tolerant bacteria have been reported to grow at low and variable temperature regime, minute amount of unfrozen water, and nutrient scarcity [6]. Unlike their mesophilic counterparts, EPS from psychrophiles provide non-pathogenic products with potential utility in food, pharmaceutical and cosmetic industries. The commercial potential of these extremophilic bacterial EPS is being investigated of late [7]. The polar regions of planet earth are among the coldest and driest. These cold tolerant microorganisms possess special physiological and biochemical characteristics [8]. Permafrost comprises of about a quarter of the total share of terrestrial soil ecosystem [9]. Exploration of permafrost microbes could lead to the discovery of novel biomolecules for biotechnological applications [10]. These psychrophilic organisms can survive in the extremities of temperature and salinity. Various bacteria can yield abundant quantities of EPS under these harsh conditions to create and maintain protective microhabitats [11]. However, very little is known regarding the biological potential of these EPS [12].

These molecules, being unexposed to the organisms of the mesophilic nature, might be capable of producing useful bioactive response. The modification of host biological processes by endogenous or exogenous products towards a pathogen-free healthy condition is known as biological response modification. Biological response modifiers (BRM) are agents, capable of altering the normal immune response, including induction of cytokines as major mechanisms of action. The development of both immunosuppressive and immunostimulating drugs, effective in immunotherapies in organ transplantation, autoimmune disorders, cancer [13], or as vaccine adjuvants, have been possible due to research on pharmacological applications of various BRMs [14]. Polysaccharides like, lentinan and other fungal β-glucans, yeast mannan fractions, and a plethora of bacterial EPSs have shown potential activity as immunomodulatory BRMs. Of late, the main thrust area in BRM research has focused on the discovery of new BRMs as well as the

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possible mechanisms of action involved behind the immunomodulatory activity shown by these compounds [15].

The report of *Sphingobacterium* spp. have been found from soil microbial community of polar [16] environment. In this study, a novel isolate from Arctic permafrost soil, *Sphingobacterium* sp. IITKGP-BTPF3 has been characterized and used to produce an exopolysaccharide. Sphingobactan was further characterized in terms of structural complexity and its capability for biological response modification. However, this probably is the first ever report on the study of bioactivity of EPS (Sphingobactan) secreted from an Arctic *Sphingobacterium* sp.

2. Materials and methods

2.1. Materials

HiAssort Carbohydrate test kit (Himedia, India), Biochemical test kit (Himedia, India), Snake Skin® Dialysis tubing 10,000 MWCO (Thermo Fischer Scientific, USA), DEAE-52 cellulose (SRL, India), Sepharose CL-6B (GE Healthcare, UK), dextran markers (2000 kDa, 500 kDa, 100 kDa, 70 kDa and 40 kDa) (Sigma-Aldrich, USA), Tris-Cl (Himedia, India), NaCl (Himedia, India), NaOH (Himedia, India), TLC Silica gel 60 F254 plates (Merck, Germany), orcinol (Himedia, India), D_2O (Merck, Germany) were purchased from various sources. Other chemicals and solvents used were of analytical grade. DMEM and RPMI-1640 media powders were procured from BD Biosciences, USA. Muller Hinton Agar, Luria Broth, Nutrient Broth were purchased from Himedia (India). Pierce LAL Chromogenic Endotoxin Quantitation Kit was purchased from Thermo-Fischer, USA.

2.2. Isolation of bacterial strain

The bacterium has been isolated from permafrost soil sample, collected from Ny-Ålesund (78°55′ N, 11°56′ E), Svalbard Island on Arctic ocean, Norway. This strain was kindly provided to us by Dr. S.M. Singh, NCAOR, Goa. The strain was maintained on Luria agar plates at 25 °C.

2.3. 16s rRNA gene sequencing and phylogeny determination

The selected bacterial strain was identified by 16S rRNA gene sequencing. Total genomic DNA was extracted from the bacterial cell [17]. Amplification of 16S rRNA gene was achieved by PCR using universal bacterial primer [18] 27F (5′-AGAGTTTGATCCTGGCT CAG-3′) and 1492R (5′-AAGGAGGTGATCCAGCC-3′). Gel eluted PCR product was purified by QIAquick gel extraction kit (QIAGEN) as per manufacturer's protocol. The amplicon was sequenced and analyzed through BLAST to obtain the closest genus match. 16s rRNA gene sequences of these relatives were used for multiple sequence alignment and generation of phylogenic tree using the MEGA5 software [19].

2.4. Synthetic media for growth of bacteria and EPS production

For optimization of variables important for EPS production in liquid culture, bacterial strain was grown in a synthetic defined medium (DM) in all the experiments, except mentioned otherwise. The medium salt base contains (per L) K_2HPO_4 – 7 g, KH_2PO_4 – 2 g, $MgSO_4{\cdot}7H_2O$ – 0.1 g, and was set at pH – 7 after addition (per L) of tryptone – 1.0 g and dextrose – 20 g, as nitrogen and carbon sources respectively.

2.5. Optimization of EPS production

Growth and EPS production were optimized by varying the fermentation temperature (4, 15, 20, 25, 30 and 37 °C), initial media pH (5, 6, 7, 8, 9, 10, 11 and 12), different carbon sources (dextrose, sucrose, mannitol, maltose and lactose) and nitrogen sources ($NaNO_3$, KNO_3, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, glutamate, peptone, tryptone, yeast extract and beef extract), taking one variable at a time, while keeping the other parameters constant. The experiments were carried out in triplicates in 250 mL Erlenmeyer flasks with 100 mL media, at 150 rpm shaking condition.

2.6. Purification of EPS from culture broth

Culture broth was centrifuged at 8000 rpm, 30 min for obtaining cell free broth. To remove the dissolved proteins by centrifugation, the cell free broth was treated with trichloroacetic acid (10% w/v) [20]. To the supernatant, ice cold isopropanol (3 volume) was added and kept at 4 °C overnight for precipitation of EPS [21–23]. EPS was recovered by centrifugation dried in hot air oven. This was further dissolved in water and dialysed using 10,000 MWCO membrane (Thermo-Fischer, USA) and then freeze dried to obtain white powdery crude EPS. This crude EPS was subjected to anion exchange chromatography through Tris-Cl (pH-7.8) pre-equilibrated DEAE-52 cellulose (SRL, India) column (18 cm × 1.8 cm) and eluted with increased step gradient of NaCl solution [23]. Fractions (5 mL per fraction) were collected and checked for eluted EPS using phenol-sulphuric acid assay of neutral carbohydrates [24]. The EPS containing fractions were dialyzed and concentrated for further purification and characterization. Pierce LAL Chromogenic Endotoxin Quantitation Kit was used for detection of any endotoxin residue whether present in the purified EPS, using manufacturers' protocol.

2.7. Characterization of EPS

2.7.1. EPS molecular weight determination

The major fraction after ion exchange elution was loaded (100 mg) onto a size exclusion column of Sepharose CL-6B (2 cm × 70 cm) and eluted with water, with a flow rate of 0.5 mL/min. All the fractions (5 mL each) were collected and tested for the presence of EPS by phenol-sulphuric acid method [24]. For the preparation of standard curve, dextran molecular weight standards (500 kDa, 100 kDa, 70 kDa and 40 kDa; Sigma-Aldrich, USA) were run through the column with identical condition. Void volume of the column was determined by running blue dextran (> 2000 kDa). A linear expression obtained by plotting log (molecular wt.) against elution volume was used to calculate the molecular weight of the purified EPS.

2.7.2. FT-IR analysis

The infrared spectrum (IR) of EPS (KBr disc method) was recorded with a Nexus-870 FTIR spectrometer (Thermo Nicolet Corporation, USA) in the range 4000–400/cm [25].

2.7.3. Determination of monomeric composition of EPS

2.7.3.1. Thin Layer Chromatography (TLC). EPS (5 mg) was hydrolyzed with 1 mL of 4 M trifluoroacetic acid (TFA) by heating at 100 °C in oil bath for 4 h [26]. Remaining acid was removed completely through co-distillation with methanol. Monosaccharide composition of TFA digested EPS was determined by TLC. Isopropanol: Ethyl acetate: acetic acid: water (4:2:2:1) were used as mobile phase on TLC Silica gel 60 F_{254} plates (MERCK, Germany) with orcinol-sulphuric acid solution as spraying reagent [27].

2.7.3.2. HPLC. TFA hydrolyzed EPS, after removal of excess acid and dissolution in chloroform, was injected into a Zorbax Carbohydrate Column in Agilent HPLC system with a mobile phase of acetonitrile:water (66:34) at a flow rate of 1.4 mL/min. The run was performed at 35 °C and the elution profile detected with RI detector.

2.7.3.3. Gas chromatography – mass spectrometry. The monosaccharide composition of the purified EPS was determined following alditol-acetate derivatization method, as described by Englyst and Cummings [28]. The monosaccharide standards were treated in a similar fashion. After hydrolysis of polysaccharide into monomeric sugars by TFA

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