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Structural and immunological studies of an exopolysaccharide from *Acinetobacter junii* BB1A

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ABSTRACT

A water-soluble heteropolysaccharide (PS) was isolated from extracellular polymeric substances (EPS) produced by a novel metal tolerant bacterium, *Acinetobacter junii* BB1A. Sugar analysis showed that the PS was composed of mannose, galactose and arabinose in a molar ratio of nearly 3:1:1. Structural characterization of the PS was carried out using methylation analysis, periodate oxidation, Smith degradation and 1D/2D NMR experiments. Methylation analysis revealed that the PS was consisted of 2,4-linked-mannopyranosyl, 3,4-linked-mannopyranosyl, 2-linked-galactopyranosyl, terminal mannopyranosyl and arabinopyranosyl residues in a relative proportion of nearly 1:1:1:1:1. Smith degradation of the PS showed the presence of hydrated glyceraldehyde containing disaccharide unit consisting of α-D-Man*p*-(1 → and → 4)-α-D-Man*p*-(1 → residues where the later was directly attached to a hydrated glyceraldehyde moiety. This polysaccharide showed significant *in vitro* splenocyte, thymocyte, and macrophage activations with optimum dose of 100 μg/mL for macrophage and 25 μg/mL both for the splenocyte and thymocyte.

1. Introduction

Extracellular polymeric substances (EPS) are metabolic products which accumulate on the bacterial cell surface (Morgan, Forster, & Evison, 1990) and serve as a protective layer for the cells against harsh environmental condition. EPS serve as carbon as well as energy source during the starvation of bacteria (Liu & Fang, 2002) and are found to compose of organic substances like polysaccharide, protein, DNA, humic acid (Wang, Liu, Yao, & Cai, 2006) out of which polysaccharide was identified as the predominant constituent (Liu & Fang, 2002). Polysaccharides in EPS show variations in sugar compositions and linkages, chain length, repeating units and substitutions in the side chains (Sutherland, 1972). Bacterial exopolysaccharides are significant because of their application as bioflocculant, bioadsorbents, heavy metal removal and drug delivery agents (Wang, Ahmed, Feng, Li, & Song, 2008) and also for their biological activities like antitumor, immunostimulatory, and anti-inflammatory activities (Arena et al., 2006; Weiner, Langille, & Quintero, 1995). Polysaccharides activate macrophages, T-helper, NK, and other effector cells and thereby activate the various cytokines (IL-2, IL-6, IL-10, and IL-12), interferon (IFN-γ), and chemokines which ultimately stimulate host's immune system. It has been observed that the molecular mass, degree of branching, conformation and chemical modification of polysaccharides significantly affect their anti-tumor and immunomodulatory activities (Bohn & BeMiller, 1995; Okazaki, Adachi, Ohno, & Yadomae, 1995). Three species of the genus *Acinetobacter* were earlier reported to produce emulsifiers made up of polysaccharides. RAG-1 emulsan produced by *A. venetianus* was reported to compose of D-galactosamine, L-galactosaminuronic acid, and a diamino, 2-deoxy *N*-acetylglucosamine (Dams-Kozlowska, Mercaldi, Panilaitis, & Kaplan, 2008). The BD4 emulsan produced by *Acinetobacter calcoaceticus* consists of a polysaccharide containing L-rhamnose, D-glucose, D-glucuronic acid, and D-mannose in molar ratio of 4:1:1:1 (Kaplan, Rosenberg, Jann, & Jann, 1985). The bioemulsan produced by *A. radioresistens* KA53 is a complex anionic polysaccharide (Navon-Venezia et al., 1995). *Acinetobacter junii* BB1A, a novel metal-tolerant bacterium isolated from Torsa River of Northern West Bengal, India, produces extracelluar polymeric substances (EPS) (Yadav et al., 2012). Chemical analysis revealed that the EPS was composed of both polysaccharide and protein. The average molecular weight of the polysaccharide (PS) fraction of EPS was ~2×10^5 Da and GC analysis of PS revealed the presence of three

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constituent sugars, mannose, galactose, and arabinose in a molar ratio of 3:1:1 as reported earlier (Yadav et al., 2012). Although there are some reports on the sugar composition of the exopolysaccharides produced by different *Acinetobacter* species, report regarding their detailed structural characterization is still absent in the literature. In the present study, detailed structural characterization of the exopolysaccharide isolated from *A. junii* BB1A was carried out along with some *in vitro* immunoactivation studies and reported herein for the first time.

2. Materials and methods

2.1. Isolation and purification

A. junii BB1A, a novel metal tolerant biofilm forming bacteria was reported earlier to produce extracellular polymeric substances (EPS) in liquid brain heart infusion (BHI) medium (Yadav et al., 2012). EPS was isolated from culture broth through centrifugation, precipitation in double volume of cold 95% ethanol followed by dialysis in a dialysis tubing cellulose membrane (D9652, Sigma–Aldrich, retaining MW >12,400 Da) against distilled water for 24 h. The dialyzed material was again centrifuged at 9587.5 × *g* for 30 min at 4 °C and the supernatant was freeze-dried to obtain crude EPS (500 mg). Protein fraction of EPS was removed by adding trichloroacetic acid (30%) followed by centrifugation and residue was discarded. Two volumes of 95% ethanol was added to the supernatant and centrifuged at 9587.5 × *g* for 20 min to obtain 400 mg of protein-free crude polysaccharide (Zhang, Liu, Wang, & Jiang, 2002). This crude polysaccharide (30 mg) was passed through Sepharose 6B gel permeation column (90 × 2.1 cm) using water as the eluant with a flow rate of 0.4 mL min^{-1} as described in the earlier report (Yadav et al., 2012). A total of 95 test tubes (2 mL each) were collected using Redifrac fraction collector and monitored spectrophotometrically (Shimadzu UV–vis spectrophotometer 1601) at 490 nm with phenol–sulfuric acid reagent (York, Darvill, McNeil, Stevenson, & Albersheim, 1986). A single fraction of purified polysaccharide (22 mg) was obtained.

2.2. Determination of optical rotation

Optical rotation of PS was measured on a Jasco Polarimeter model P-1020 at 31.1 °C.

2.3. Absolute configuration of the monosaccharide

The method used was based on Gerwig, Kamerling, and Vliegenthart (1978). After the hydrolysis of PS (1 mg) by TFA, the acid was removed by co-distillation with water. A solution of 250 μL of 0.625 M HCl in R-(−)-2-butanol was added into it, and the mixture was heated at 80 °C for 16 h. After butanolysis, the solution was neutralized with Ag_2CO_3 and the supernatant solution was concentrated under reduced pressure at 45 °C. The residue was dried for 12 h *in vacua* over P_2O_5, and then treated with hexamethyldisilazane–chlorotrimethylsilane–pyridine (0.1 ml, 1:1:5) for 30 min at room temperature. The products were analyzed by GC using a capillary column (SPB-1, 30 m × 0.26 mm) with a temperature program (3 °C min^{-1}) from 150 to 210 °C. The (−)-2-butyl-2,3,4,6-tetra-O-TMS-glycosides obtained were identified by comparison with those prepared from the D- and L-enantiomers of the monosaccharide.

2.4. Methylation analysis

PS (4.0 mg) was methylated using the procedure described by Ciucanu and Kerek (1984) and the product was isolated by making a partition between $CHCl_3$ and water (5:2, v/v). The methylated biopolymer was hydrolyzed with 90% HCOOH (1 mL) at 100 °C for 1 h and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed product was reduced with $NaBH_4$ (9 mg) followed by acidification with dilute CH_3COOH, and then co-distillation with CH_3OH to remove excess boric acid. The reduced sugars (alditol) were acetylated with 1:1 pyridine–Ac_2O in a boiling water bath for 1 h to give alditol acetates. The alditol acetates of the methylated sugars were analyzed by GC–MS. GC–MS analysis was performed on Shimadzu GC–MS Model QP-2010 Plus automatic system, using ZB-5MS capillary column (30 m × 0.25 mm). The program was isothermal at 150 °C; hold time 5 min, with a temperature gradient of 2 °C min^{-1} up to a final temperature of 200 °C.

2.5. Periodate oxidation and Smith degradation study

PS (25 mg) was added to 7.5 mL 0.1 M sodium metaperiodate solution and the mixture was kept for 48 h in the dark at 4 °C. The excess periodate was destroyed by adding ethylene glycol (5.0 mL) and the solution was dialyzed against distilled water for 2 h. The volume of the dialyzed material was concentrated to 2–3 mL. This material was reduced with $NaBH_4$, 12 h, neutralized with 50% AcOH, and dialyzed with distilled water and finally freeze dried (14.0 mg). The periodate-reduced material was divided into three portions. One portion (1.5 mg) was hydrolyzed with 2 M CF_3COOH (1 mL) at 100 °C for 18 h, used for alditol acetate preparation and analyzed by GC. The second portion (2.0 mg) was methylated by the method of Ciucanu and Kerek (1984), followed by preparation of alditol acetates which were analyzed by GC–MS. Smith degradation experiment was performed with the third portion (10.0 mg). The mild hydrolysis of the periodate oxidized–reduced material was performed by the addition of 0.5 M CF_3COOH for 15 h at 25 °C to destroy the residues of oxidized sugars attached to the polysaccharide chain. The excess acid was removed by repeated freeze drying. The material was further purified by passing through a Sephadex G-25 column, kept over P_2O_5 in vacuum for several days and finally used for ^{13}C NMR studies.

2.6. NMR studies

PS was dried over P_2O_5 in vacuum for several days and then deuterium exchanged five times, followed by lyophilization with D_2O (99.96%. atom ^2H, Aldrich) (Dueñas-Chaso et al., 1997). Then ^1H NMR and ^{13}C NMR experiments were performed with a Bruker Avance DPX-500 instrument at 30 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.70) using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992) using acetone as internal standard fixing methyl proton signal at δ 2.225. ^{13}C NMR experiment of PS was carried out taking acetone as the internal standard, fixing the methyl carbon signal at δ 31.45. The 2D-(DQF-COSY) NMR experiment was carried out using standard pulse sequence at 30 °C. The TOCSY experiment was recorded at a mixing time of 60–300 ms. The NOESY and ROESY mixing delay were 300 ms. Delay time in the HMBC experiment was 80 ms.

2.7. Study of immunoenhancing activities of PS

2.7.1. Preparation of LPS free PS

Prior to the immunoactivation studies, LPS free PS was prepared in order to rule out the contribution of LPS which may contaminate in the course of isolation and purification process. The PS was passed through polymyxin-B agarose matrix (P1411, Sigma and Aldrich, USA) packed in 2 mL column (1 cm × 2 cm) with a flow rate of 0.5 mL min^{-1}. It was equilibrated with 10 mM phosphate buffer, pH 7.4. The bacterial lipopolysaccharides (LPS) were bound

to the matrix and the unbound LPS free PS (LFPS) were eluted and collected for immunoactivation studies.

2.7.2. Limulus amebocyte lysate (LAL) test

Limulus amebocyte lysate (LAL) test was carried out *in vitro* for detection of bacterial endotoxin. The test was performed using gel clot technique (Liu et al., 2009). Limulus amebocyte lysate (LAL) (G2125, sensitivity: 0.125 EU/mL) was purchased from Quantum Biotech, Mumbai, India. The control standard endotoxin (CSE) (code E0125) and water (code W1004) for the bacterial endotoxin test (BET) were provided by Quantum Biotech, Mumbai, India. Four tubes were taken, each containing 0.1 mL of LAL reagent. In two tubes, 0.1 mL LFPS aqueous solution were added, meanwhile 0.1 mL BET water and 0.1 mL CSE were added to the rest two tubes as negative control and positive control, respectively. All tubes were incubated for 1 h at 37 °C. After the test tube was inverted 180° slowly, it is positive (+) if the gel in tube is not deformed and does not slip from the wall and a negative (−) test is characterized in absence of a gel or by the formation of a viscous mass which does not hold when the tube is inverted. Test is invalid when positive control is (−) or negative control is (+).

2.7.3. Test for macrophage activity by nitric oxide assay

RAW 264.7 growing in Dulbecco's modified Eagle's medium (DMEM) at 37 °C was seeded in 96 well flat bottom tissue culture plate at 5×10^5 cells/mL concentration (180 μL) (Maiti et al., 2011). Cells were left overnight for attachment and treated with different concentrations (12.5, 25, 50, 100 or 200 μg/mL) of LFPS. After 48 h of treatment, culture supernatant of each well was collected and NO production was estimated using Griess Reagent (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) (Green et al., 1982). Lipopolysaccharide (LPS) (L6511 of *Salmonella enterica* serotype typhimurium, Sigma, St. Louis, USA, 4 μg/mL) was used as positive control and soluble starch (Merck, India, 100 μg/mL) as negative control (Maity et al., 2013).

2.7.4. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus were prepared from the normal mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS). The suspension was centrifuged to obtain cell pellet. The contaminating red blood cells (RBC) were removed by hemolytic Gey's solution. After washing two times in HBSS the cells were resuspended in complete RPMI (Roswell Park Memorial Institute) medium. Cell concentration was adjusted to 1×10^6 cells/mL and the viability of splenocytes and thymocytes (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96-well flat-bottom tissue culture plates and incubated with 20 μL of various concentrations of LFPS (12.5, 25, 50, 100, and 200 μg/mL). PBS (Phosphate Buffer Saline, 10 mM, pH 7.4) was taken as carrier control and soluble starch (Merck, India, 100 μg/mL) as negative control (Maity et al., 2013). LPS (L6511 of *S. enterica* serotype typhimurium, Sigma, St. Louis, USA, 4 μg/mL) served as positive control for splenocytes and Concanavalin A (Himedia, 10 μg/mL) for thymocytes. All cultures were set up at 37 °C for 72 h in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes (% Splenocyte Proliferation Index or % SPI) and thymocytes (% Thymocyte Proliferation Index or %TPI) were checked by MTT assay method (Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006). The data are reported as the mean ± standard deviation of seven different observations and compared against PBS control (Green et al., 1982; Maiti et al., 2008).

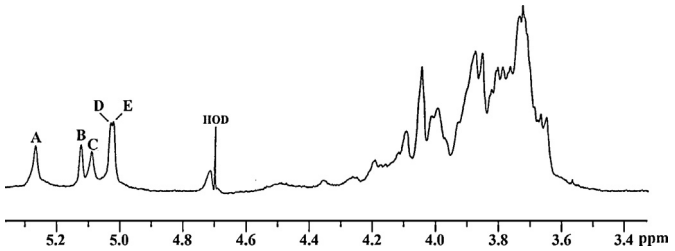


Fig. 1. ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A. Acetone was taken as the internal standard, fixing the methyl proton signal at δ 2.225.

3. Results and discussion

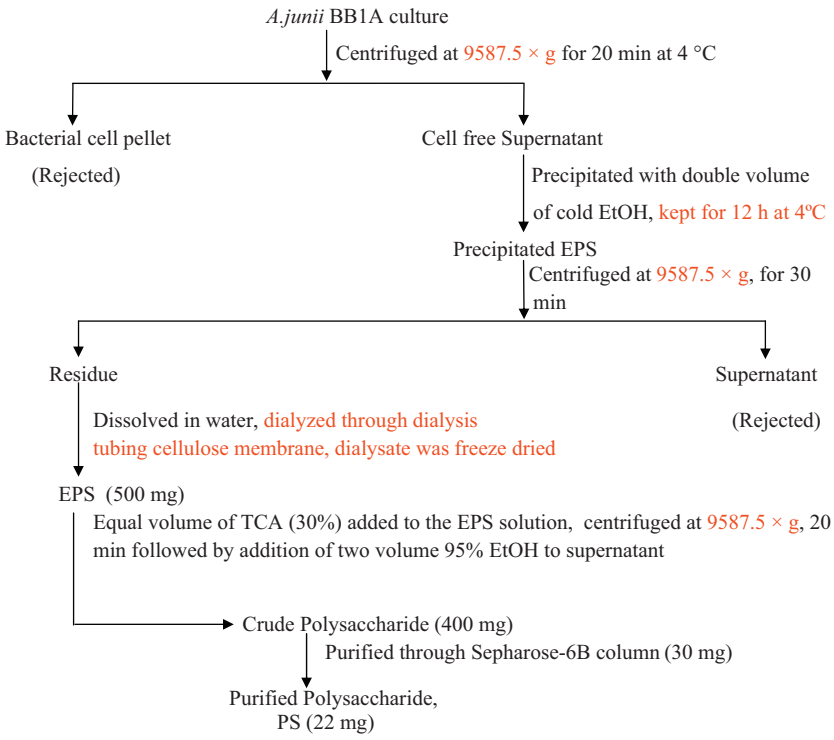
3.1. Purification and chemical analysis of PS

A single fraction was obtained after fractionating water soluble crude polysaccharide (30 mg) through Sepharose 6B column. The fraction (test tubes 8–18) was collected and freeze dried, yielding 22 mg of purified polysaccharide (PS). The isolation and purification steps are summarized in Scheme 1. The PS showed a specific rotation of $[\alpha]_D^{31}$ +15.8 (*c* 0.1, water) and was composed of mannose, galactose and arabinose in a molar ratio of nearly 3:1:1 as reported earlier (Yadav et al., 2012). Determination of absolute configuration of the monosaccharides showed that mannose and galactose were present in D and arabinose in L configuration. The GC–MS analysis of partially methylated alditol acetates revealed the presence of 2,4-linked-mannopyranosyl, 3,4-linked-mannopyranosyl, 2-linked-galactopyranosyl, terminal mannopyranosyl and arabinopyranosyl residues in a relative proportion of approximately 1:1:1:1:1 (Table 1a). GC analysis of alditol acetates of the periodate-oxidized (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965), $NaBH_4$-reduced PS was found to contain D-mannose only. GC-MS analysis of periodate-oxidized, reduced, and methylated PS showed the presence of 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-mannitol, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-mannitol in a molar ratio of nearly 1:1 (Table 1b), indicating the 2,4- and 3,4-linked-mannopyranosyl residues remained unaffected during periodate-oxidation. This observation further confirmed the mode of linkages present in the PS.

3.2. Structural analysis of PS

The ^{1}H NMR spectrum (Fig. 1) of PS at 30 °C showed the presence of five signals in the anomeric region at δ 5.27, 5.13, 5.09, 5.02, and 5.01 in a ratio of nearly 1:1:1:1:1. In ^{13}C spectrum (Fig. 2) four signals were found at δ 102.2, 102.1, 100.1, and 98.3 in a ratio of nearly 1:2:1:1. The five anomeric proton signals corresponded to five sugar residues designated as **A**, **B**, **C, D** and **E** according to their decreasing proton chemical shift values. In the HSQC spectrum (Fig. 3), the anomeric proton signal of **A** at δ 5.27 was correlated to the carbon signal at δ 100.1. The anomeric proton signal at δ 5.13 was correlated to the carbon signals at δ 102.2 and assigned to anomeric carbon of residue **B**. The anomeric proton signal of **C** at δ 5.09 was correlated to the carbon signal at δ 98.3. Again, the anomeric proton signal at δ 5.02 and 5.01 were correlated to the carbon signal at δ 102.1 corresponding to the anomeric carbon of both residues **D** and **E**. All the ^{1}H and ^{13}C signals (Table 1c) were assigned from DQF-COSY, TOCSY, and HSQC experiments. The proton coupling constants were measured from DQF-COSY experiment.

The residue **A** was assigned for the galacto configuration for its large $J_{H\text{-}2,H\text{-}3}$ coupling constant (~8 Hz) and relatively small $J_{H\text{-}3,H\text{-}4}$ coupling constant (~3 Hz). The α-configuration of residue



Scheme 1. Flow diagram of isolation and purification of the heteropolysaccharide (PS) from EPS produced by *A. junii* BB1A.

Table 1a
GC–MS analysis of methylated heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A.

Residue	Methylated sugars	Molar ratio	Linkage type	Major mass fragments (*m*/*z*)
A	3,4,6-Me_3-Gal	1	→2)-D-Gal*p*-(1→	43,57,74,87,99,**129**,145,173,200
B	2,3,4,6-Me_4-Man	1	D-Man*p*-(1→	43,59,73,87,**101**,117,129,145,161,205
C	2,3,4-Me_3-Ara	1	L-Ara*p*-(1→	43,58,71,87,**101**,117,129,131,161
D	3,6-Me_2-Man	1	→2,4)-D-Man*p*-(1→	43,59,74,87,99,**129**,143,173,189,203,233
E	2,6-Me_2-Man	1	→3,4)-D-Man*p*-(1→	43,58,74,87,101,**117**,129,143,159,173,189,233

Table 1b
GC–MS analysis of periodate oxidized methylated heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A.

Residue	Methylated sugars	Molar ratio	Linkage type	Major mass fragments (*m*/*z*)
F	3,6-Me_2-Man	1	→2,4)-D-Man*p*-(1→	43,59,74,87,99,**129**,143,173,189,203,233
G	2,6-Me_2-Man	1	→3,4)-D-Man*p*-(1→	43,58,74,87,101,**117**,129,143,159,173,189,233

A was assigned from $J_{H\text{-}1,H\text{-}2}$ coupling constant (~3 Hz) and $J_{C\text{-}1,H\text{-}1}$ of (~170 Hz) and from its anomeric proton (δ 5.27) and carbon (δ 100.1) signals. The downfield shift of C-2 (δ 77.8) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **A** was a (1 → 2)-linked-D-galactopyranosyl residue.

Residue **B** was assigned to Man*p* which showed large coupling constants of $J_{H\text{-}3,H\text{-}4}$ (~7 Hz) and $J_{H\text{-}4,H\text{-}5}$ (~9 Hz). The anomeric proton signal at δ 5.13 and the coupling constant values of $J_{H\text{-}1,H\text{-}2}$ (~1.6 Hz), $J_{H\text{-}2,H\text{-}3}$ (~3.5 Hz) and $J_{C\text{-}1,H\text{-}1}$ (~170 Hz) clearly indicated that the residue **B** was α-linked mannopyranosyl moiety. The carbon chemical shifts of residue **B** from C-1 to C-6 corresponded

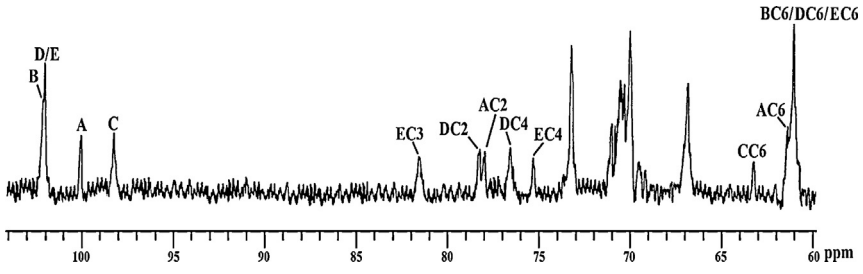


Fig. 2. ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A. Acetone was taken as the internal standard, fixing the methyl carbon signal at δ 31.45.

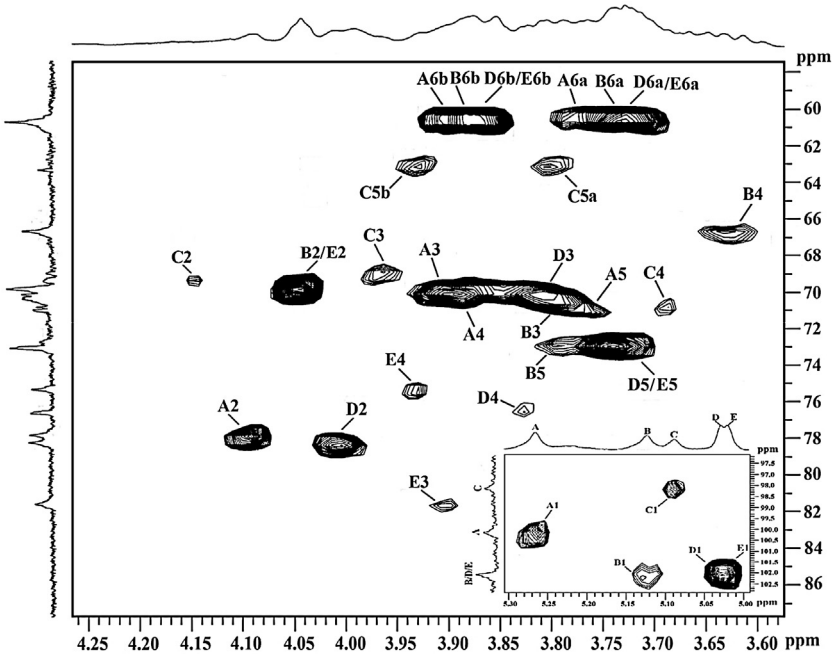


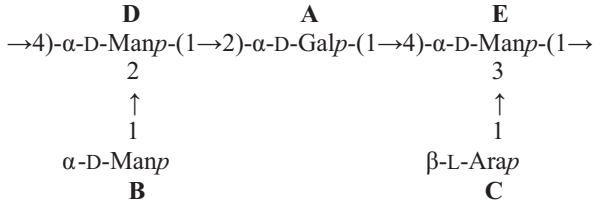
Fig. 3. Part of HSQC spectrum of heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A. The annotation A1 refers to AH1/AC1 cross peak, B1 refers to BH1/BH2 cross peak and so on. Inset shows the HSQC spectrum of the anomeric region.

nearly to the standard values of methyl glycoside of α-D-mannose indicating residue **B** was terminal α-D-mannopyranosyl moiety.

Residue **C** was assigned to Ara*p* which showed a large coupling constant $J_{H\text{-}2,H\text{-}3}$ (~8.2 Hz), $J_{H\text{-}3,H\text{-}4}$ (~5 Hz), relatively small coupling constant $J_{H\text{-}1,H\text{-}2}$ and two H-5 signals (δ 3.80 and 3.93). The anomeric proton chemical shift for residue **C** at δ 5.09 and carbon chemical shift at δ 98.3, $J_{H\text{-}1,C\text{-}1}$ ~170 Hz (measured from proton coupled ^{13}C spectra) indicated that L-arabinose is β configured at the anomeric center. The carbon chemical shifts of residue **C** from C-1 to C-5 corresponded nearly to the standard values of methyl glycosides of β-L-arabinose indicating that the residue **C** was non-reducing end Ara*p*.

The manno configuration of residues **D** and **E** was supported from large coupling constants of $J_{H\text{-}3,H\text{-}4}$ (~7 Hz) and $J_{H\text{-}4,H\text{-}5}$ (~9 Hz). The anomeric proton signals at δ 5.02 and 5.01 and the coupling constant values of $J_{H\text{-}1,H\text{-}2}$ (~1.6 Hz), $J_{H\text{-}2,H\text{-}3}$ (~3.5 Hz) and $J_{C\text{-}1,H\text{-}1}$ (~170 Hz) clearly indicated that both **D** and **E** residues were α-linked mannopyranosyl moieties. The downfield shift of C-2 (δ 78.0) and C-4 (δ 76.6) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **D** was a 2,4-linked-D-mannopyranosyl residue. The downfield shift of C-3 (δ 81.8) and C-4 (δ 75.4) of residue **E** with respect to standard values of methyl glycosides (Agrawal, 1992, Rinaudo & Vincendon, 1982) indicated that it was a 3,4-linked-D-mannopyranosyl residue.

The sequence of glycosyl residues was established from ROESY as well as NOESY (not shown) experiments. In ROESY experiment (Fig. 4a, Table 1 in supplementary data), the inter-residual contacts from $\mathbf{A}_{H\text{-}1}/\mathbf{D}_{H\text{-}1}$ and $\mathbf{E}_{H\text{-}4}$; $\mathbf{B}_{H\text{-}1}/\mathbf{D}_{H\text{-}2}$; $\mathbf{C}_{H\text{-}1}/\mathbf{E}_{H\text{-}3}$; $\mathbf{D}_{H\text{-}1}/\mathbf{A}_{H\text{-}1}$, $\mathbf{A}_{H\text{-}2}$ and $\mathbf{E}_{H\text{-}1}/\mathbf{D}_{H\text{-}4}$ along with other intra-residual contacts were observed. A long range HMBC experiment was carried out to confirm the connectivites obtained from ROESY experiment. Inter-residual cross-peaks, $\mathbf{A}_{H\text{-}1}/\mathbf{E}_{C\text{-}4}$, $\mathbf{A}_{C\text{-}1}/\mathbf{E}_{H\text{-}4}$; $\mathbf{B}_{H\text{-}1}/\mathbf{D}_{C\text{-}2}$; $\mathbf{B}_{C\text{-}1}/\mathbf{D}_{H\text{-}2}$; $\mathbf{C}_{H\text{-}1}/\mathbf{E}_{C\text{-}3}$, $\mathbf{C}_{C\text{-}1}/\mathbf{E}_{H\text{-}3}$; $\mathbf{D}_{H\text{-}1}/\mathbf{A}_{C\text{-}2}$, $\mathbf{D}_{C\text{-}1}/\mathbf{A}_{H\text{-}2}$; $\mathbf{E}_{H\text{-}1}/\mathbf{D}_{C\text{-}4}$, $\mathbf{E}_{C\text{-}1}/\mathbf{D}_{H\text{-}4}$ along with some intra-residual cross peaks were also observed (Fig. 4b, Table 2 in supplementary data). Thus, the HMBC and ROESY connectivities clearly supported the presence of the pentasaccharide repeating unit in the heteropolysaccharide (PS) isolated from EPS produced by *A. Junii* BB1A as:



3.3. Smith degradation study

Smith degradation was carried out with the PS where a disaccharide containing hydrated glyceraldehyde moiety is produced (Scheme 1 in supplementary data) and the product was analyzed by ^{13}C NMR spectroscopy (Table 2, Fig. 1 in supplementary data) to confirm further the sequence of the sugar residues present in the repeating unit. The ^{13}C NMR spectrum of Smith-degraded PS showed two anomeric carbon signals at δ 103.2 and 103.1 corresponding to α-D-Man*p*-(1 → (**F**) and → 4)-α-D-Man*p*-(1 → (**G**) residues, respectively indicating a disaccharide unit produced during Smith degradation. The carbon signal at δ 76.2 clearly indicated the presence of 4-linked α-D-Man*p* unit (**G**) which was generated from → 2,4)-α-D-Man*p*-(1 → residue (**D**) and the → 3,4)-α-D-Man*p*-(1 → residue (**E**) was converted to nonreducing end α-D-Man*p* unit (**F**) during degradation. During oxidation two aldehyde groups were generated at C-3 and C-4 positions of → 2)-α-D-Gal*p*-(1 → residue (**A**) which on reduction followed by hydrolysis produced free glycerol and disaccharide containing glyceraldehyde which in water may exist in hydrated form (H). The carbon signals at δ 90.5, 72.1, and 62.6 were corresponded to C-1, C-2, and C-3 of the hydrated glyceraldehyde moiety (H). Hence, the structure of hydrated glyceraldehyde containing disaccharide unit obtained from heteropolysaccharide (PS) after Smith degradation was established as:

Table 1c
The ^{1}H[a] and ^{13}C[b] NMR chemical shifts of the sugar residues of heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A in D_2O at 30 °C.

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6
→2)-α-D-Gal*p*-(1→	5.27	4.10	3.91	3.89	3.77	3.78[c], 3.86[c]
A	100.1	77.8	70.0	70.0	71.0	61.1
α-D-Man*p*-(1→	5.13	4.04	3.79	3.63	3.80	3.72[c], 3.87[c]
B	102.2	70.0	70.2	66.7	73.0	61.1
β-L-Ara*p*-(1→	5.09	4.15	3.96	3.69	3.80[c], 3.93 [d]	-
C	98.3	69.3	69.1	70.9	63.2	
→2,4)-α-D-Man*p*-(1→	5.02	4.01	3.81	3.83	3.74	3.74[c], 3.88[c]
D	102.1	78.0	70.1	76.6	73.1	61.1
→3,4)-α-D-Man*p*-(1→	5.01	4.04	3.90	3.93	3.74	3.74[c], 3.88[c]
E	102.1	70.0	81.8	75.4	73.1	61.1

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.7 at 30 °C.
[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone, fixing the methyl carbon signal at δ 31.45 and proton signal at δ 2.225 using D_2O as the solvent.
[c] Interchangeable.

α-D-Man*p*-(1→4)-α-D-Man*p*-(1→2)-CH(CH$_2$OH)CH(OH)$_2$
F **G** **H**

Therefore, Smith degradation results further confirmed the repeating unit present in the heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A.

3.4. Immunostimulating properties of LFPS

A negative (−) LAL test indicated that LFPS which was obtained after passing the PS through polymixin-B matrix was free from bacterial endotoxin. Macrophage activation by LFPS was observed *in vitro*. Upon treatment with different concentrations of LFPS, enhanced production of NO was observed in a dose dependent manner with optimum production of 20.00 μM NO per 5×10^5

Table 2
The ^{13}C NMR[a] chemical shifts of disaccharide unit obtained after Smith-degradation of heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A in D_2O at 30 °C.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Man*p*-(1→ **F**	103.2	70.8	70.2	66.7	73.2	61.2
→4)-α-D-Man*p*-(1→ **G**	103.1	70.8	69.8	76.2	73.2	61.2
1 2 3 (HO)$_2$HC-CH-CH$_2$OH ↑ **H**	90.5	72.1	62.6	–	–	–

[a] Values of the ^{13}C chemical shifts were recorded with reference to acetone, fixing the methyl carbon signal at δ 31.45.

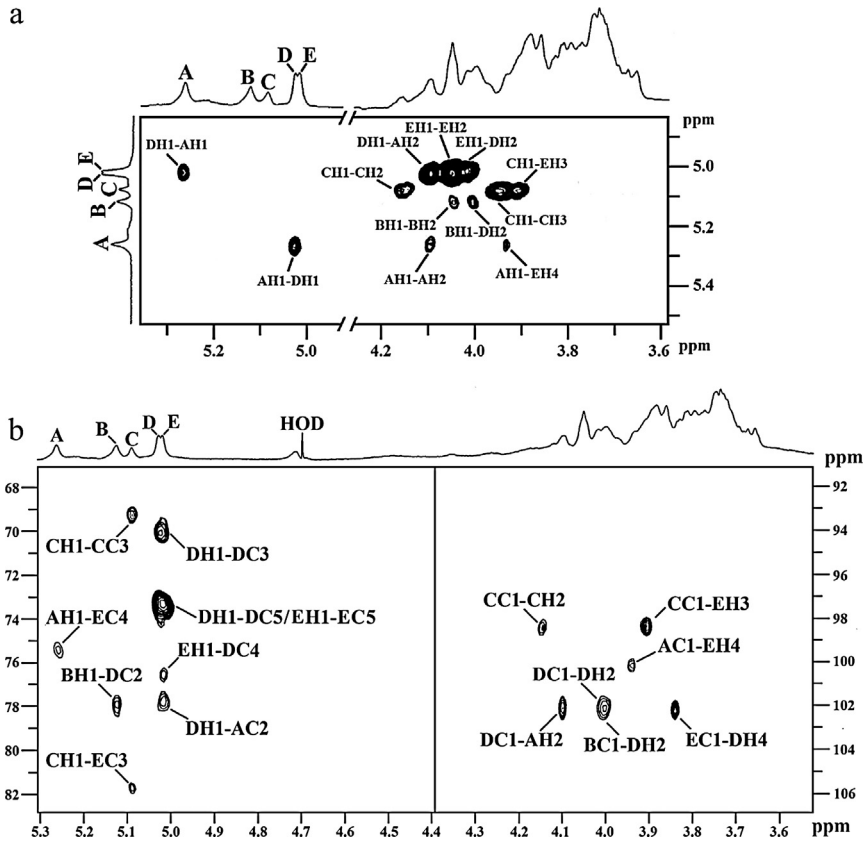


Fig. 4. (a) Part of ROESY spectrum of heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A. The ROESY mixing time was 300 ms. (b) Part of HMBC spectrum of heteropolysaccharide (PS) isolated from EPS produced by *A. junii* BB1A. The delay time in the HMBC experiment was 80 ms.

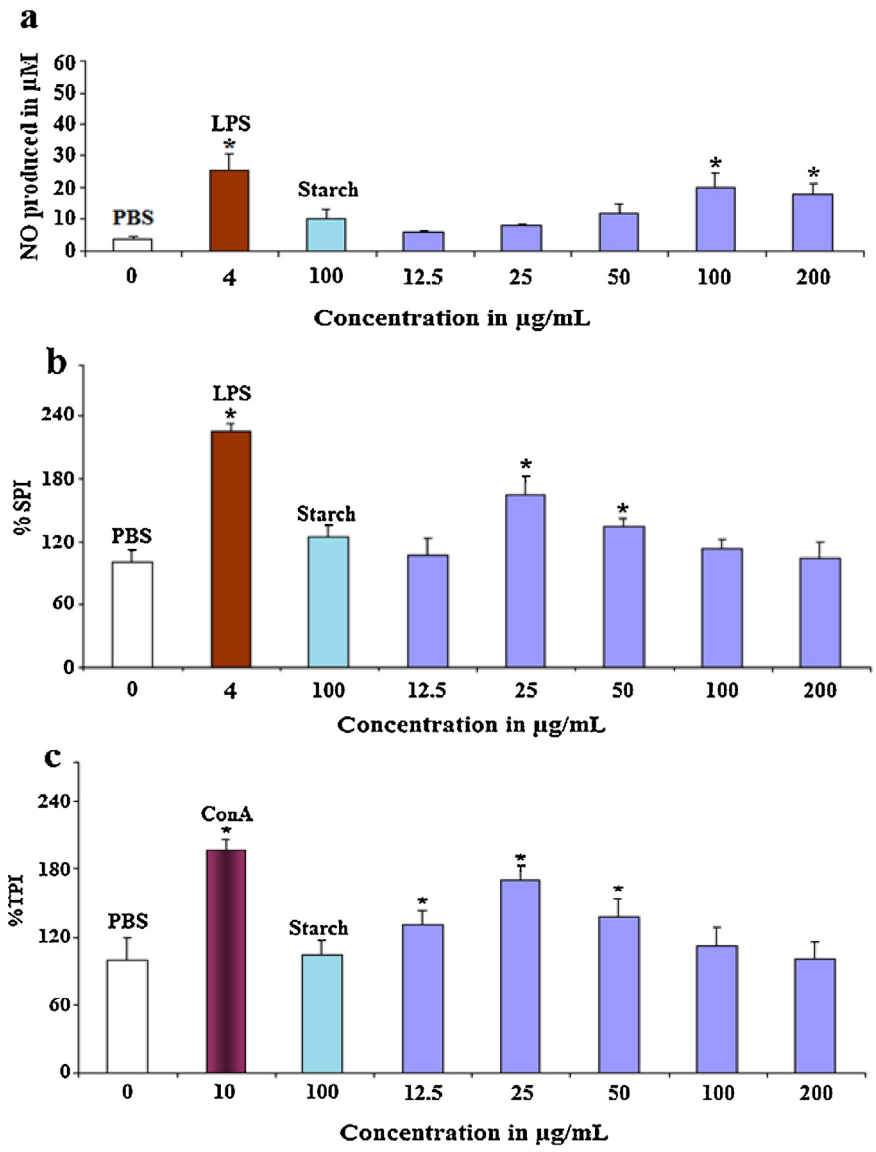


Fig. 5. (a) *In vitro* activation of macrophage stimulated with different concentrations of LPS free PS (LFPS) in terms of NO production. Effect of different concentrations of LPS free PS (LFPS) on proliferation of (b) splenocyte and (c) thymocyte (*statistically significant differences compared to PBS control).

macrophages at an effective dose of 100 μg/mL (Fig. 5a). Hence, the effective dose of LFPS for macrophage activation was 100 μg/mL.

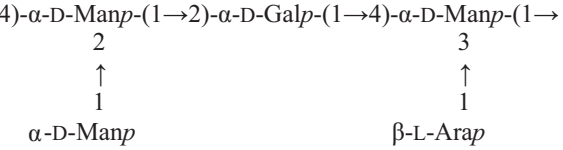
Proliferation of splenocytes and thymocytes is an indicator of immunoactivation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with LFPS by the MTT assay method (Sarangi et al., 2006). LFPS was tested to stimulate splenocytes and thymocytes and the results are shown in Fig. 5b and c, respectively. Both splenocyte and thymocyte proliferation index were found to be maximum at 25 μg/mL of LFPS, above or below which it decreases. Hence, it can be concluded that 25 μg/mL is the optimum concentration of LFPS both for splenocyte and thymocyte proliferation. Recently, an exopolysaccharide produced by *Bacillus licheniformis* 8-37-0-1 was found significantly to stimulate the proliferation of splenocytes in a dose-dependent manner at concentrations from 50 μg/mL to 800 μg/mL (Liu et al., 2010).

From the above results it is noted that optimum concentration of LFPS for NO production was 100 μg/mL whereas, for splenocyte and thymocyte proliferation, the optimum concentration of LFPS was 25 μg/mL. It has been reported that polysaccharides interact with cell surface receptor molecules like dectin-1, CR3, and TLRs (Gantner, Simmons, Canavera, Akira, & Underhill, 2003) and activate macrophages, T-helper, NK, and other effector cells. The cellular responses like proliferation and NO production depend on intensity of signal produced in upstream at cell membrane receptor level which further depends on cell type, the number of receptor molecules present at the cell surface and nature of the stimulant (here polysaccharide) (Bohn & BeMiller, 1995; Okazaki et al., 1995; Ohno, Miura, Nakajima, & Yadomae, 2000). These factors may influence on the results obtained with PS, which induces maximum NO production at four times higher concentration than requires for splenocyte and thymocyte proliferation. However, further studies on mechanism of action of PS are needed to get more insight of its action.

4. Conclusions

A heteropolysaccharide (PS) was isolated from extracellular polymeric substances (EPS) produced by a novel metal tolerant bacterium, *A. junii* BB1A. On the basis of chemical analysis and NMR

studies the structure of the repeating unit of this heteropolysaccharide was established as:



The PS showed significant *in vitro* macrophage, splenocyte and thymocyte activations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.09.018.

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