Carbohydrate Polymers 90 (2012) 683–689

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structural elucidation and bioactivity of a novel exopolysaccharide from endophytic *Fusarium solani* SD5

Subhadip Mahapatra, Debdulal Banerjee*

Microbiology Laboratory, Department of Botany and Forestry, Vidyasagar University, Midnapore 721102, West Bengal, India

ARTICLE INFO

Article history:
Received 20 March 2012
Received in revised form 21 May 2012
Accepted 26 May 2012
Available online 4 June 2012

Keywords:
Anti allergic activity
Anti inflammatory activity
Endophytic *Fusarium solani* SD5
Exopolysaccharide
GLC–MS
NMR

ABSTRACT

A bioactive exopolysaccharide [EPS (PS-I)], having $M_w \sim 1.87 \times 10^5$ Da was produced by submerged culture of an endophytic fungus *Fusarium solani* SD5. Structural elucidation of the EPS (PS-I) was carried out by a series of experiments. Result indicates the presence of terminal α-L-rhamnopyranosyl, (1→2)-α-L-rhamnopyranosyl, (1→4)-β-D-galactopyranosyl, (1→4,6)-β-D-galactopyranosyl moieties in a molar ratio of nearly 1:1:3:1. TEM image showed fibril structure of the EPS with a diameter of approximately 1 nm. Melting point range of the EPS was found 172–178 °C. The isolated PS-I exhibit *in vitro* anti inflammatory and anti allergic activity. EPS (1000 μg/ml) protects 55% erythrocytes from hypotonic solution induced membrane lysis. Compound 48/80 induced mast cell degranulation was also protected by 56% with 100 μg/ml EPS.

1. Introduction

Two hundred years ago Henri Braconnot discovered chitin, first carbohydrate polymer from edible mushrooms (Muzzarelli et al., 2012), but till date carbohydrate research does not get much attention like genomes and proteins (Hurtley, Service, & Szuromi, 2001). Research in glycoscience especially fungal exopolysaccharide (EPS) had extensively been increasing for the last two decades due to their centuple applications in industry as well as medicine and pharmacy (Lee et al., 2003; Lee, Cho, Kim, Hong, & Yoo, 1996; Muzzarelli et al., 2012); such as, thickening and stabilizing agents (Lee et al., 2003), immunostimulating and antitumor agents (Lee et al., 2003), component of various cosmetics (Czop & Kay, 1991), immunotherapeutic agent for cancer treatment (Lee et al., 2003), and antianemics (Podkolzin, Dontsov, Sychev, Kobeleva, & Yu Kharchenko, 1996). Among the fungal EPS producers, basidiomycetes and soil duteromycetes were extensively studied. Now, researchers are in search of new fungus with no previous application in glycobiology. Fungus residing in the healthy plant tissues for at least a part of its life cycle, without causing apparent harm to its host, is called endophytic fungus (Mahapatra & Banerjee, 2009). This endophytic fungus which is considered as such type of under explored trove useful for isolation of microorganism is able to produce exopolysaccharide, interesting both scientifically and biotechnologically. At present, only a few literatures are available regarding EPS production by endophytic fungi (Banerjee, Jana, & Mahapatra, 2009; Chen et al., 2011; Li et al., 2011). The EPS produced from endophytic fungi were mainly studied for their antioxidant activity (Chen et al., 2011) and elicitor activity for the production of important plant secondary metabolites (Li et al., 2011). Together with the knowledge of physicochemical properties, the structures of EPS are also very important for understanding their physiological activities. However, according to the best of our knowledge no literature is available relevant to structural characterization of exopolysaccharide having anti inflammatory and anti allergic activity (http://www.ncbi.nlm.nih.gov/pubmed?).

Therefore, the present investigation is an attempt to examine and illustrate the structural characterization of exopolysaccharide produced by an endophytic fungus, *Fusarium solani* SD5 by ^{1}H NMR, ^{13}C NMR and GLC–MS. This EPS has also been assessed for *in vitro* anti inflammatory and anti allergic activity.

2. Materials and methods

2.1. Microorganism

F. solani SD5 was isolated as an endophyte from the stem of *Alstonia scholaris*. The organism was maintained and stored on potato dextrose agar (PDA) slants at 25 °C and 4 °C respectively.

* Corresponding author. Tel.: +91 03222 276554x430; fax: +91 03222 275329.
E-mail addresses: debu33@gmail.com, debu33@yahoo.com, db@mail.vidyasagar.ac.in (D. Banerjee).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.05.097

2.2. Identification of endophyte

The reproductive structure of the organism was studied under optical compound microscope and scanning electron microscope (SEM). Finally, identification was confirmed by rRNA based molecular techniques. In brief, fungal genomic DNA was isolated and quality was evaluated on 1.2% agarose gel, a single band of high-molecular weight DNA has been observed. A polymerase chain reaction (PCR) was performed using ITS1 (5′-TCC GTA GGT GAA CTT TGC GG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers. A single discrete PCR amplicon band of 700 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 605 bp was used for further analysis. Sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were compared to the GenBank database using BLAST software on the NCBI website (http://ncbi.nlm.nih.gov/BLAST/). Fifteen sequences including our SD5 were selected and aligned using multiple alignment software program Clustal W and the phylogenetic tree was constructed using MEGA 4 (Tamura, Dudley, Nei, & Kumar, 2007).

2.3. Production of exopolysaccharides (EPS)

Experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of the potato dextrose broth (PDB) medium, which were inoculated (1 cm^2 mycelial growth of selected endophytic fungi) and incubated at 25 °C for 14 days.

2.4. Isolation and estimation of EPS

The culture was filtered and centrifuged at 10,000 × *g* to separate fungal biomass. The supernatant was concentrated in rotary evaporator (Eyela N-1000, Japan) under low pressure at 40 °C. Absolute ethanol was added to the filtrate (5:1, v/v) and after 24 h at 4 °C the precipitate was recovered by centrifugation at 10,000 × *g* for 10 min, and dialyzed in a cellulose membrane (molecular weight cut off 10,000) against distilled water for 24 h. The concentration of the EPS was estimated spectrophotomatrically by phenol sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Then isolated biopolymer was vacuum-dried and stored in desiccator for further investigation.

2.5. Molecular weight determination

The average molecular weight of the exopolysaccharide was determined by a gel-chromatographic technique performed on a Sepharose-6B gel filtration column (65 cm × 2 cm) eluted with distilled water at a flow rate of 0.4 ml/min in a Eyela automated fraction collector (model no. DC-1000). The elution volume of EPS was plotted on the standard calibration curve prepared by plotting the elution volume of standard dextrans (T-40, T-70 and T-200 having M_w 40,000 Da, 70,000 Da and 200,000 Da, respectively) against the logarithm of their respective molecular weights and the molecular weight of EPS was determined.

2.6. Protein estimation

Presence of protein was determined according to Lowry, Rosenbrough, Farr, and Randall (1951) using bovine serum albumin as standard.

2.7. Paper chromatography study

The exopolysaccharide (1.5 mg) was hydrolyzed by treatment with 2 M trifluoroacetic acid (CF_3COOH) (1 ml) in a round bottomed flask at 100 °C for 18 h. The excess acid was removed by co-distillation with water. One part of the hydrolyzed material was used for paper chromatographic analysis. Paper chromatographic studies were performed on Whatmann nos. 1, 3 mm sheets. Solvent systems were used as BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase) and EtOAc–pyridine–H_2O (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution (Hoffman, Lindberg, & Svensson, 1972).

2.8. Monosaccharide analysis

The remaining part of the previously hydrolyzed material was then used for preparing alditol acetates. The alditol acetates of sugars were prepared by the method followed by Das et al. (2008) and analyzed by GLC (Gas-liquid chromatography) performed with a Hewlett-Packard 5810 gas chromatography equipped with a flame-ionization detector (FID). The instrument was fitted with a glass column (1.8 m × 6 mm) packed with 3% ECNSS-M on Gas Chrom Q (100–120 mesh) at 170 °C and 1% OV-225 on Gas Chrom Q (100–120 mesh) at 170 °C and data were analyzed using HP software. Sugar identification was done by comparison with the retention time of alditol acetates of standard reference sugars. Quantification was carried out from the peak area.

2.9. Methylation analysis

The exopolysaccharide was methylated using the procedure described by Ciucanu and Kerek (1984). The procedure for product isolation and the conversion of monosaccharides into their corresponding methylated alditol acetates was done following the method used by Das, Mondal, Maiti, Roy, and Islam (2009). The sugar linkages of the constituent methylated alditol acetates were analyzed by GLC using two above said columns and also by GLC–MS (Gas-liquid chromatography–mass spectrometry) analysis, performed on Shimadzu GLC–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 using software GCMSsolution.

2.10. Optical rotation

Optical rotation was measured on a Perkin-Elmer model-241 MC polarimeter at 589 nm, cell length 100 mm, 25 °C.

2.11. NMR spectroscopy

The ^1H and ^{13}C NMR experiments were recorded at 500 MHz, on a Bruker Avance DPX-500 spectrometer using a 5-mm broadband probe. For NMR measurements PS was dried in vacuum over P_2O_5 for several days, and was then exchanged with deuterium (Deunas Chaso et al., 1997) by lyophilizing with D_2O for several times. The deuterium-exchanged polysaccharide (5 mg) was dissolved in 0.7 ml of D_2O (99.96% atom ^2H, Aldrich). The ^1H and ^{13}C NMR spectra were recorded at 27 °C. Acetone was used as the internal standard (31.05 ppm) for the ^{13}C spectrum. The ^1H NMR spectrum was recorded by fixing the HOD signal at 4.79 ppm. Data were recorded using Bruker Topspin3.1 software.

2.12. Transmission electron microscopic (TEM) study

The purified EPS was examined under proper condition in a transmission electron microscope (JEOL-JEM-2010 HRTEM). For

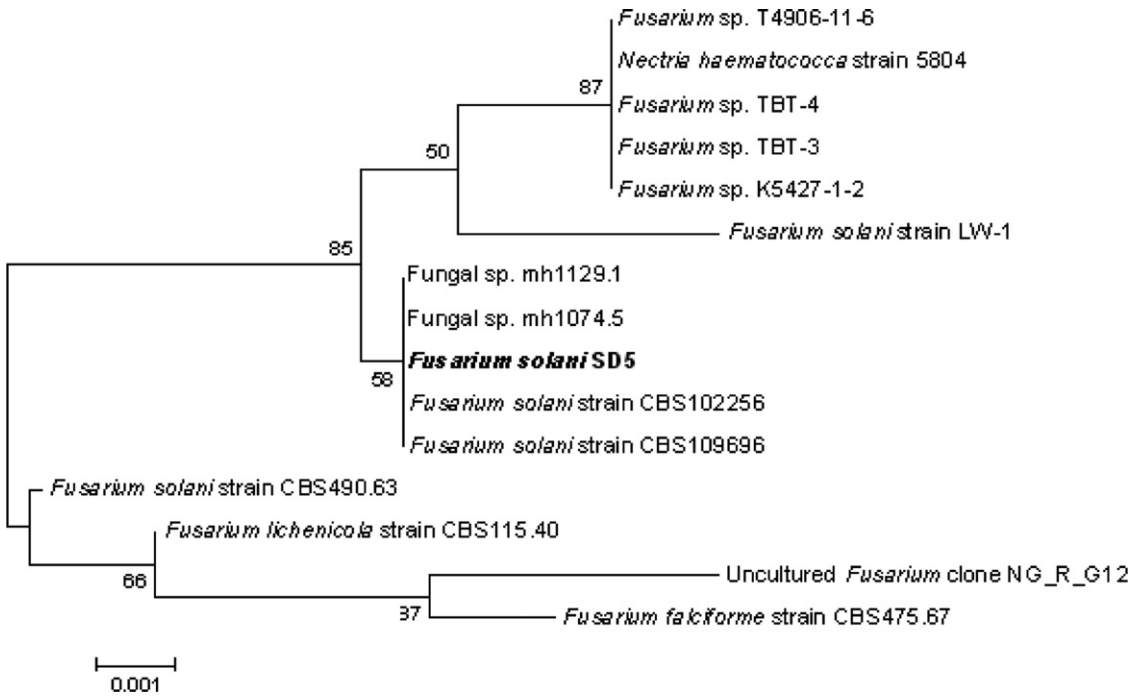


Fig. 1. Evolutionary relationships of *F. solani* SD5 and some related fungi.

TEM analysis samples were prepared by drop casting the polysaccharide suspension on a carbon coated copper grid.

2.13. Melting point determination

The purified, dried EPS was transferred into a capillary tube and melting point was determined using a programmable melting point apparatus (Veego, India; Model-VMP-PM).

2.14. Animals for bioactivity study

Experiments were performed using Male Wistar rats weighing 200–225 g. The animals were fed standard pellet diet with vitamins, antibiotic and were given *ad libitum* access to water and housed in polypropylene cages (Tarson) with 12 h light:dark cycle. The animals were allowed to acclimatize for one week. The animals used did not show any sign of malignancy or other pathological processes. Animals were maintained in accordance with the guidelines of the ethical committee of Vidyasagar University, Midnapore, West Bengal, India.

2.14.1. Membrane stabilization activity

In vitro membrane stabilization was performed using the method of hypotonicity induced rat erythrocyte hemolysis described by Shinde et al. (1999). Whole blood of rats was collected by retro-orbital puncture using heparinized syringe. The whole blood samples were centrifuged at 3000 rpm for 10 min. The erythrocytes pellet was collected and washed by re-suspending with isotonic buffered solution (154 mM NaCl in 10 mM phosphate buffer pH 7.4). The procedure repeated three times and each time erythrocytes were centrifuged at 3000 rpm for 10 min. The test sample consisted of varying concentrations of EPS (10, 100 and 1000 μg/ml) or INDO (10 μg/ml) (GenPharma International Pvt. Ltd., India) and 0.50 ml of stock erythrocyte suspension in 4.0 ml of hyposaline solution. The control sample consisted of 0.5 ml of stock erythrocyte suspension with hypotonic buffered saline solution. The reaction mixtures were incubated at 56 ± 1 °C for 30 min and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 540 nm. The inhibition percentage of erythrocyte haemolysis was calculated according to the method of Shinde et al. (1999).

$$\text{Inhibition of erythrocyte hemolysis (\%)} = \left[\frac{\text{absorbance1} - \text{absorbance2}}{\text{absorbance1}}\right] \times 100$$

where absorbance1, absorbance of control and absorbance2, absorbance of test samples.

2.14.2. Mast cells protecting activity

The protection of mast degranulation induced C 48/80 (Sigma–Aldrich, USA) was performed using the method describe by Norton (1954). The overnight fasted male Wistar rats were anesthetize with excess ether and open the whole abdomen to expose the intestine. The intestinal mesenteries were collected in Ringer–Locke solution and the mesenteries were cut into small pieces. The pieces of mesentery were placed in different petri dishes consisting of different concentrations of EPS (10, 100 and 1000 μg/ml) or DSCG (10 μg/ml) (Cipla, India) prepared in Ringer Locke solution. The petri-dishes were incubated with toxicant C 48/80 (0.8 μg/ml) at 37 °C for 30 min. Two sets of control were prepared by incubating pieces mesentery with or without toxicant C 48/80 in Ringer–Locke solution. The tissues were placed on a clean microscopic slide and remove the fatty layer carefully. The trimmed tissues were stained with 4% formaldehyde solution containing 0.1% O-toludine blue (Spectrochem Pvt. Ltd., India) for 30 min. The tissues were then de-stained by successive washing with acetone and xylene for 5 min. The stained mesentery pieces were examined under digital light microscope at 100× magnification. The mast cell was considered as degranulated if 4–5 granules were present around the cells. Percentages of degranulated and intact mast cells were calculated on the basis of counting 100 mast cells for each sample. For each set

of test sample including control 6–8 pieces of mesentery were observed.

2.14.3. Statistical analysis

All the results were expressed as mean ± SEM of the number of the experiments. Statistical significance was performed by one-way ANOVA followed by Bonferroni's multiple comparison or Dunnett's multiple comparison tests whichever applicable. The data analysis was performed using Graph Pad Prism software.

3. Results and discussion

3.1. Identification of Fusarium sp. SD5

The organism appeared to have a whitish mycelium with heavily intertwining hyphae. Cultural and reproductive characteristics of SD5 showed that the organism had septate, hyaline and wide spread hyphae; linear asexual conidiospore; conidia terminal, single, fusiform, and curved (data not shown here).

The rDNA gene sequence data of fungi SD5 were deposited as entry JQ657824 in GenBank. A BLAST search of the database indicated a close genetic relationship with other isolates of *Fusarium* sp. The evolutionary history of *F. solani* SD5 was inferred using the neighbor-joining method (Saitou & Nei, 1987). An optimal phylogenic tree with the sum of branch length = 0.02203415 was constructed (Fig. 1). The percentage of replicate trees, in which the associated taxa were clustered together in the bootstrap test (500 replicates), was illustrated next to the branches (Felsenstein, 1985). The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura, Nei, & Kumar, 2004) and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 580 positions in the final dataset.

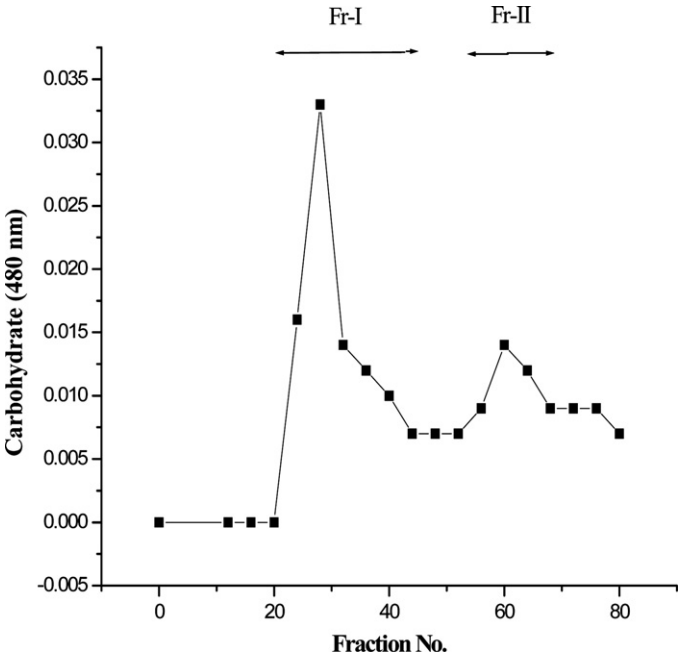


Fig. 2. Gel filtration chromatograms of the exopolysaccharides produced by the endophytic fungus *F. solani* SD5. The crude polysaccharides obtained from the endophytic fungus were applied to a Sepharose-6B column and eluted fractions containing the polysaccharides were pooled and Fr-I was named EPS (PS-I).

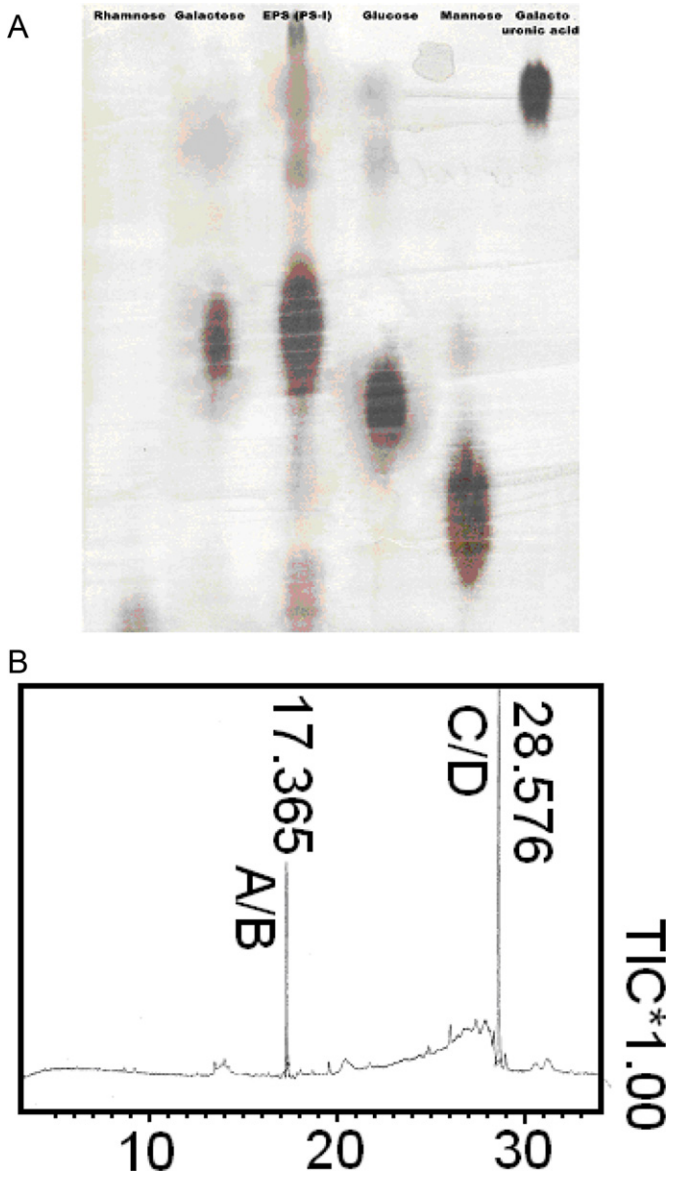


Fig. 3. (A) Paper chromatographic analysis of hydrolyzed EPS (PS-I) isolated from *F. solani* SD5. (B) GLC chromatogram of the EPS (PS-I) isolated from *F. solani* SD5. A/B indicates rhamnose and C/D indicates galactose.

Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

3.2. Purification and molecular weight determination of EPS

The endophytic fungi *F. solani* SD5 was grown in potato dextrose broth for 14 days. The fermented broth was filtered, centrifuged, and EPS was precipitated in ethanol. The precipitated materials on dialysis followed by freeze drying yielded crude polysaccharide. The water-soluble crude polysaccharide was finally purified by gel filtration chromatography using a Sepharose-6B column. Two fractions of EPSs (Fr-I and Fr-II) were collected (Fig. 2). Fr-I and Fr-II were eluted between 20–35 and 50–65 tubes, respectively. The absence of protein moiety indicates the purity of polysaccharides. The major fraction, Fr-I [EPS (PS-I)] was considered for further investigation. The molecular weight (Hara, Kiho, Tanaka, & Ukai, 1982) of the homogeneous EPS (PS-I) was estimated from a calibration curve prepared with standard dextrans (data are not

Table 1
GLC–MS data for the methylated sugar moieties of the EPS (PS-I) isolated from *F. solani* SD5.

Methylated sugars	Linkage types	Molar ratio	Major fragments (*m*/*z*)
3,4-Me_2-6-deoxy-Rha*p*	→2)-(-L-Rha*p*-(1→	1	43, 59, 71, 72, 87, 89, 101, 113, 115, 129, 131, 189
2,3,4-Me_3-6-deoxy-Rha*p*	Terminal	1	43, 59, 72, 89, 101, 115, 117, 131, 161, 175
2,3-Me_2-Gal*p*	→4,6)-β-D-Gal*p*-(1→	1	43, 45, 57, 71, 75, 85, 87, 99, 101, 117, 127, 129, 143, 159, 161, 187, 202
2,3,6-Me_3-Gal*p*	→4)-β-D-Gal*p*-(1→	3	43, 45, 59, 71, 87, 99, 101, 113, 117, 129, 131, 142, 161, 173, 233

shown here) as $\sim 1.87 \times 10^5$ Da. It showed a specific rotation of $[\alpha]_D^{25}$ + 18.6 (*c* 0.094 gm/100 ml, water).

3.3. Analytical

Paper chromatographic analysis of the hydrolyzed product showed the presence of galactose and rhamnose (Fig. 3A). The GLC analysis of the alditol acetates of the EPS (PS-I) showed the presence of galactose and rhamnose in the molar ratio of nearly 2:1 (Fig. 3B). The absolute configuration of the monosaccharides was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). The sugar residues, galactose and rhamnose had D and L configuration, respectively.

The mode of linkages of the purified EPS (PS-I) was determined by methylation analysis using Ciucanu and Kerek method (1984). The GLC and GLC–MS of alditol acetates of the methylated product showed the presence of 1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methyl-L-rhamnitol; 1,2,5-tri-*O*-acetyl-6-deoxy-3,4-di-*O*-methyl-L-rhamnitol; 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-galactitol in a molar ratio of nearly 1:1:3:1. This result indicated the presence of terminal L-rhamnopyranosyl, (1→2)-L-rhamnopyranosyl, (1→4)-D-galactopyranosyl, (1→4,6)-D-galactopyranosyl moieties in a molar ratio of nearly 1:1:3:1 (Table 1).

The ^{1}H NMR spectrum (500 MHz, Fig. 4A) of the EPS (PS-I) recorded in D_2O at 27 °C showed the presence of three signals in the anomeric region at 5.20, 5.10, and 4.61 ppm (Table 2) in a ratio of nearly 1:1:4. The sugar residues were designated as **A**, **B**, and **C** according to their decreasing anomeric proton chemical shift values. The ^{13}C NMR (500 MHz) spectrum (Fig. 4B) at 27 °C showed that three anomeric signals appeared at 100.8, 100.3, and 104.3 ppm (Table 2) in a molar ratio of nearly 1:1:4. The sugar residues **A** and **B** were α-L-rhamnopyranosyl moieties and **C** is β-D-galactopyranosyl moiety (discussed later). But the experimental data of methylation analysis showed that there were two types of galactopyranosyl residues. Hence, the other galactopyranosyl residue was taken as **D** which had the same anomeric ^{1}H and ^{13}C values like the residue **C**.

The anomeric signals of residue **A** and **B** at 5.2 and 5.1 ppm, respectively, indicated that both were α-linked sugar residues (Roy et al., 2007). Both the residues were assigned as Rha*p* due to the signals for an exocyclic —CH_3 group and also the two ^{1}H signals at 1.29 and 1.27 ppm and also the ^{13}C signals at 17.4 and 16.7 ppm, respectively. The downfield chemical shift of C-2 of residue **A** at 78.1 ppm from standard methyl glycosides (Agrawal, 1992) indicated that it was (1→2)-linked. The carbon chemical shifts of **B** from C-1 to C-6 corresponded nearly to the standard values for methyl glycosides. Thus considering the experimental data of both methylation analysis and NMR experiment it is concluded that residue **A** was (1→2)-α-L-rhamnopyranosyl and **B** was terminal α-L-rhamnopyranosyl moieties.

The anomeric ^{1}H signal of both the residues **C** and **D** at 4.61 ppm indicated that they were β-linked (Agrawal, 1992; Ojha et al.,

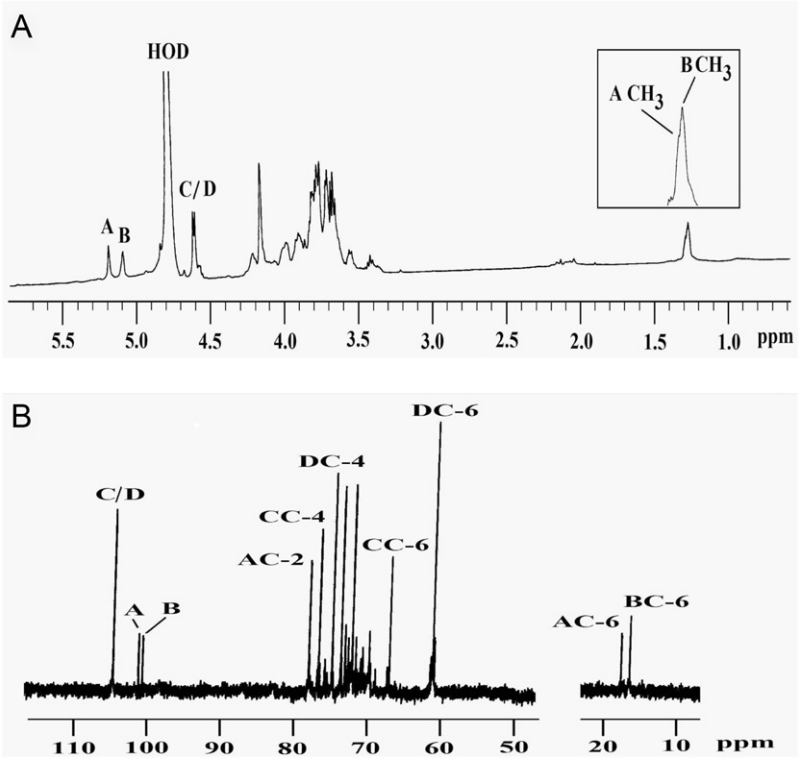


Fig. 4. (A) ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of the EPS (PS-I) isolated from *F. solani* SD5. (B) ^{13}C NMR spectrum (500 MHz, D_2O, 27 °C) of the EPS (PS-I) isolated from *F. solani* SD5.

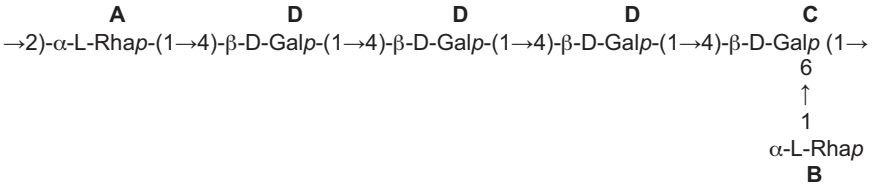
Table 2
The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts for the EPS (PS-I) isolated from *F. solani* SD5 in D_2O at 27 °C.

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)-(-L-Rha*p*-(1→	5.20	4.01	3.78	3.56	3.87	1.29
A	100.8	78.1	70.3	73.2	69.4	17.4
(-L-Rha*p*-(1→	5.10	3.93	3.81	3.54	3.89	1.27
B	100.3	71.2	71.8	74.3	74.8	16.7
→4,6)-(-D-Gal*p*-(1→	4.61	3.44	3.51	3.91	3.69	3.76[c], 4.17[d]
C	104.3	71.3	72.7	76.2	75.1	67.4
→4)-(-D-Gal*p*-(1→	4.61	3.43	3.57	3.93	3.66	3.67[c], 3.72[d]
D	104.3	71.3	73.2	74.8	76.1	60.9

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.79 ppm at 27 °C.
[b] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.
[c] Interchangeable.
[d] Interchangeable.

2009). The anomeric carbon chemical shift at 104.3 ppm also indicated that **C** and **D** were β-linked anomer (Agrawal, 1992; Ojha et al., 2009). Hence from the anomeric ^{1}H and ^{13}C values and also the GLC analyses, it is concluded that both **C** and **D** were β-D-galactopyranosyl moieties. The downfield chemical shift of C-4 and C-6 of residue **C** at 76.2 and 67.3 ppm, respectively, indicated that it was (1→4,6)-β-D-galactopyranosyl moiety (Agrawal, 1992; Ojha et al., 2009). The downfield chemical shift of C-4 of residue **D** at 74.8 ppm indicated that it was (1→4)-β-D-galactopyranosyl moiety (Agrawal, 1992; Ojha et al., 2009). Thus considering the experimental data of both methylation analysis and NMR experiment it is concluded that **C** was (1→4,6)-β-D-galactopyranosyl moiety and **D** was (1→4)-β-D-galactopyranosyl moiety.

Thus on the basis of these experiments the structure of the repeating unit present in the exopolysaccharide (PS-I), isolated from *F. solani* SD5 is established as:



The M_w of isolated EPS (PS-I) was detected as ~1.87×10^5 Da; each repeating unit of this EPS (PS-I) contained four galactose and two rhamnose units. So, it can be concluded that the colossal catena structure of the isolated EPS (PS-I) contained nearly 178 repeating units.

3.4. Transmission electron microscopy (TEM) analysis of PS-I

Water soluble exopolysaccharide formed by *F. solani* SD5 was studied under transmission electron microscopy (TEM). The result showed that the EPS consisted of linear fibrils with a diameter of approximately 1 nm (Fig. 5).

3.5. Melting temperature determination

The melting point range was found to be 172–178 °C using the melting point apparatus. This result indirectly supports the presence of galactose probably as a major constituent of the EPS (PS-I). On the other hand, high melting temperature of this EPS (PS-I), showed a beneficial storage facility if it is used as a drug in future.

3.6. Bioactivity study

In vitro membrane stabilizing activity was performed using hypotonic solution induced hemolysis of rat erythrocytes. It was observed that the EPS (PS-I) from *F. solani* SD5 provided significant membrane protection against hyposaline induced lyses of erythrocytes and the result was comparable with known standard drug indomethacin (Table 3). At dose concentrations of 10, 100 and 1000 μg/ml of EPS (PS-I), the percentages of membrane stabilization were calculated as 27.46 ± 4.22%, 45.08 ± 2.80% and 55.05 ± 5.68% ($P < 0.05$), respectively, whereas 43.72 ± 2.02% ($P < 0.05$) of membrane stabilization was observed in case of indomethacin. So, the EPS (PS-I) could play a key role in protection of cellular membrane from toxic substances and interfere in early phase inflammatory reactions *via* inhibiting the formation of inflammatory mediators (Shinde et al., 1999). Not only that, the erythrocyte membrane is analogous to the lysosomal membrane, and its stabilization implies, the EPS (PS-I) may as well stabilize lysosomal membrane. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents, which caused further tissue inflammation (Saleem et al., 2011).

In vitro mast cells protection study on rat mesenteric mast cells showed that the Compound 48/80 significantly degranulated mast cells (92.21 ± 0.57%, $P < 0.001$) at dose concentration of 4 μg/ml in comparison to the control (2.19 ± 0.3%)(Table 3). In this experiment it was observed that the isolated EPS (PS-I) inhibited the Compound 48/80 mediated mast cells degranulation in a dose dependent way. The EPS at a dose of 10 μg/ml reduced degranulation percentage

Table 3
Membrane stabilization activity and mast cell protection activity of the EPS (PS-I) isolated from *F. solani* SD5. Statistical analysis was done through one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and Bonferroni's multiple comparison test, respectively. Indomethacin *vs.* EPS ($c = P < 0.05$); C 48/80 *vs.* DSCG ($a = P < 0.001$) and EPS treated groups ($a = P < 0.001$).

Test samples	Concentration (μg/ml)	Erythrocyte membrane stabilization (%)	Mast cell degranulation (%)
Indomethacin	10	43.72 ± 2.02	–
EPS	10	27.46 ± 4.22	61.63 ± 3.71
	100	45.08 ± 2.80	40.26 ± 1.17
	1000	55.05 ± 5.68	30.22 ± 5.77
Compound 48/80	4	–	92.21 ± 0.57
Disodium cromoglycate	10	–	15.49 ± 3.68

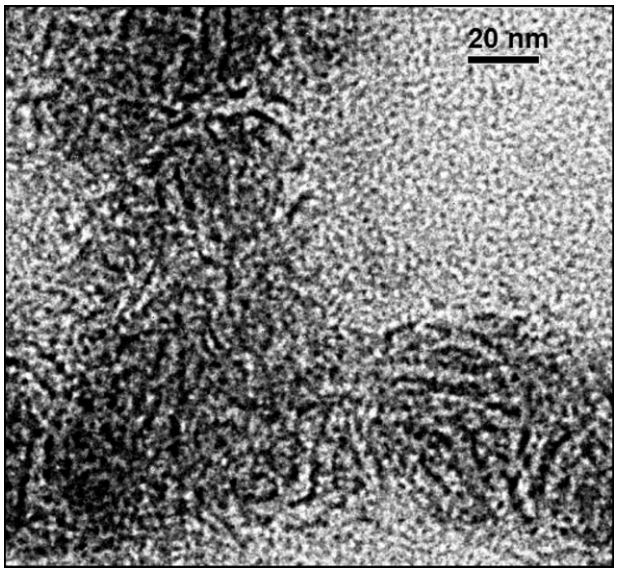


Fig. 5. Transmission electron microscopy (TEM) image of the purified EPS (PS-I) isolated from *F. solani* SD5 (bar = 20 nm).

of mast cells into 61.63 ± 3.71%, which was comparable with the standard mast cells protector DSCG (15.49 ± 3.68%, 10 μg/ml) with significance of $P < 0.001$. The EPS (PS-I) at 100 μg/ml reduced the degranulation upto 40.26 ± 1.17% ($P < 0.001$) which indicated that the EPS (100 μg/ml) protected about 56% of mast cells from degranulation by Compound 48/80. Mast cell disruption is an important initial event in the development of type I allergic reactions. Compound 48/80 is a powerful histamine releasing agent from mast cells. In this experiment it was found that purified EPS (PS-I) of endophytic *F. solani* SD5 offered significant mast cells stabilization activity. As a consequence it inhibited histamine release and thus possessed anti allergic activity.

4. Conclusions

From the present investigation we have found that an endophytic *F. solani* SD5 produced the exopolysaccharide (PS-I), a heteropolysaccharide of galactose and rhamnose; rhamno galactan, containing a hexa saccharide repeating unit with a novel structure. The EPS (PS-I) has significant mast cell stabilizing and membrane protective activities. Thus, this exopolysaccharide may offer significant effects for preclusion of inflammatory and allergic conditions. However, this study is a preamble; further studies should be performed on optimization of the EPS production in submerged fermentation and anti inflammatory activity analysis *in vivo*.

Acknowledgments

University Grants Commission (UGC), New Delhi, is thankfully acknowledged for financial assistance in major research project [F no. 40-131/2011 (SR)]. The author (S.M.) is grateful to UGC, New Delhi, India for providing fellowship [UGC-RFSMS, sanction no. F-4-3/2006(BSR)/11-114/2008(BSR)].

References

Agrawal, P. K. (1992). NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry*, *31*, 3307–3330.

Banerjee, D., Jana, M., & Mahapatra, S. (2009). Production of exopolysaccharide by endophytic *Stemphylium* sp. *Micologia Aplicada International*, *21*, 57–62.

Chen, Y., Mao, W., Tao, H., Zhu, W., Qi, X., Chen, Y., et al. (2011). Structural characterization and antioxidant properties of an exopolysaccharide produced by the mangrove endophytic fungus *Aspergillus* sp. Y16. *Bioresource Technology*, *102*, 8179–8184.

Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *131*, 209–217.

Czop, J. K., & Kay, J. (1991). Isolation and characterization of β-glucan receptors on human mononuclear phagocytes. *Journal of Experimental Medicine*, *173*, 1511–1520.

Das, D., Maiti, D., Chandra, K., Mondal, S., Ojha, A. K., Roy, S. K., et al. (2008). NMR and MALDI-TOFMS analysis of a heteroglycan isolated from hot water extract of edible mushroom, *Volvariella bombycina*. *Carbohydrate Research*, *343*, 2258–2265.

Das, D., Mondal, S., Maiti, D., Roy, S. K., & Islam, S. S. (2009). Structural characterization of dietary fiber of green chalcumra (*Benincasa hispida*) fruit by NMR spectroscopic analysis. *Natural Product Communication*, *4*, 547–552.

Deunas Chaso, M. T., Rodriguez-Carvajal, M. A., Mateo, P. T., Franko-Rodriguez, G., Espartero, J. L., Iribas, A. I., et al. (1997). Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus* 2.6. *Carbohydrate Research*, *303*, 453–458.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*, 783–791.

Gerwig, G. J., Kamerling, J. P., & Vliegenthart, J. F. G. (1978). Determination of the D and L configuration of neutral monosaccharides by high-resolution capillary g.l.c. *Carbohydrate Research*, *62*, 349–357.

Hara, C., Kiho, T., Tanaka, Y., & Ukai, S. (1982). Anti-inflammatory activity and conformational behavior of a branched (1→3)-β-D-glucan from an alkaline extract of *Dictyophora indusiata* Fisch. *Carbohydrate Research*, *110*, 77–87.

Hoffman, J., Lindberg, B., & Svensson, S. (1972). Determination of the anomeric configuration of sugar residues in acetylated oligo- and polysaccharides by oxidation with chromium trioxide in acetic acid. *Acta Chemica Scandinavica*, *26*, 661–666.

Hurtley, S., Service, R., & Szuromi, P. (2001). Cinderella's coach is ready. *Science*, *291*, 2337.

Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., Hwang, H. J., et al. (2003). Biological activities of the exopolysaccharides produced from submerged culture of the edible basidiomycete *Grifola frondosa*. *Enzyme and Microbial Technology*, *32*, 574–581.

Lee, J. H., Cho, S. M., Kim, H. M., Hong, N. D., & Yoo, I. D. (1996). Immunostimulating activity of polysaccharides from mycelia of *Phellinus linteus* grown under different culture conditions. *Journal of Microbial Biotechnology*, *6*, 52–55.

Li, P., Mou, Y., Shan, T., Xu, J., Li, Y., Lu, S., et al. (2011). Effects of polysaccharide elicitors from endophytic *Fusarium oxysporium* Dzf17 on growth and diosgenin production in cell suspension culture of *Dioscorea zingiberensis*. *Molecules*, *16*, 9003–9016.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, *193*, 255–275.

Mahapatra, S., & Banerjee, D. (2009). Extracellular tannase production by endophytic *Hyalopus* sp. *Journal of General and Applied Microbiology*, *55*, 255–259.

Muzzarelli, R. R. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate Polymers*, *87*, 995–1012.

Norton, S. (1954). Quantitative determination of mast cell fragmentation by compound 48/80. *Brazilian Journal of Pharmacology and Chemotherapy*, *9*, 494–497.

Ojha, A. K., Chandra, K., Ghosh, K., Bhunia, B., Maiti, T. K., & Islam, S. S. (2009). Structural analysis of an immunoenhancing heteropolysaccharide isolated from the green (unripe) fruits of *Solenum melongena* (Brinjal). *Carbohydrate Research*, *344*, 2357–2363.

Podkolzin, A. A., Dontsov, V. I., Sychev, I. A., Kobeleva, G., & Yu Kharchenko, O. N. (1996). Immunomodulating, antianemic, and adaptogenic effects of polysaccharides from plaster clover (*Melilotus officinalis*). *Bulletin of Experimental Biology and Medicine*, *121*, 597–599.

Roy, S. K., Chandra, K., Ghosh, K., Mondal, S., Maiti, D., Ojha, A. K., et al. (2007). Structural investigation of a heteropolysaccharide isolated from the pods (fruits) of *Moringa oleifera* (Sajina). *Carbohydrate Research*, *342*, 2380–2389.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406–425.

Saleem, T. K. M., Azeem, A. K., Dilip, C., Sankar, C., Prasanth, N. V., & Duraisami, R. (2011). Anti-inflammatory activity of the leaf extracts of *Gendarussa Vulgaris* Nees. *Asian Pacific Journal of Tropical Biomedicine*, *1*, 147–149.

Shinde, U. A., Phadke, A. S., Nair, A. M., Mungantiwar, A. A., Dikshit, V. J., & Saraf, V. O. (1999). Membrane stabilizing activity – A possible mechanism of action for the anti-inflammatory activity of *Cedrus deodara* wood oil. *Fitoterapia*, *70*, 251–257.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*, 1596–1599.

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 11030–11103.