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Production, Purification and Characterization of Exopolysaccharide from *Fomitopsis feei*

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Original Research Article

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Abstract: Production of exopolysaccharide from *Fomitopsis feei* was screened under nine different production media. Common production medium was found to be good for the best production and after isolation, purification by DEAE cellulose column chromatography and the purified exopolysaccharide was characterized using H1, C13 NMR, FTIR, GC-MS analyses and found to be a proteopolysaccharide.
Keywords: *Fomitopsis feei*, production, purification, characterization, exopolysaccharides.

INTRODUCTION

Different methods in production and purification of polysaccharide resulted in various forms, water-soluble, water-insoluble, neutral and acidic polysaccharide. Polysaccharides extracted are further purified via dialysis, fractional precipitation, ion-exchange chromatography, gel filtration and affinity chromatography. Likewise, many reports have demonstrated that polysaccharide extracted by different solvents greatly influence their biological activities [1-3]. Polysaccharides extracted from mushroom sources include fruiting body, mycelia and broth (mycelia-free). Most of the polysaccharides isolated from mushrooms are glucans with various types of glycosidic linkages. The structural compositions of polysaccharide greatly influence the biological activities. *Fomitopsis feei* showed good exopolysaccharide production among the other mushrooms when screened for exopolysaccharide production (data not shown). It is a brown rot fungus belongs to Fomitopsidaceae family. Hence, in the present study production, purification and characterization of polysaccharide from *Fomitopsis feei* broth culture was researched.

MATERIALS AND METHODS
Production in different media

Fomitopsis feei is a brown rot fungus isolated from Pakhal forest, Warangal district, Telangana State, India, during rainy season. It was identified phylogenetically using 28S rDNA analysis. For the production of exopolysaccharides, nine types of production media viz., Beta glucan production medium (1), GPCS medium (Glucose, polypeptone, cane sugar, soybean oil – 2), Fermentation medium (3), YMPG (Yeast extract, malt extract, peptone, glucose – 4), SGP medium (Sucrose, glucose, peptone- 5), Fungal growth medium (6), Standard polysaccharide production medium (7), Mushroom complete medium (8) and Common production medium (9) were screened and the production was carried out under still and shake conditions (150 rpm) for both 7 and 14 days of incubation at 28°C after the inoculation of each type of media (25 mL) with single 6 mm disc of *Fomitopsis feei*.

Isolation of exopolysaccharides

Crude polysaccharide was precipitated by centrifugation with isopropanol from 14 days old culture broth of *F.feei* in 1:4 ratio. Dry crude 1g of extra cellular polysaccharide was redissolved in 100 mL distilled water (at 25°C for two hours). Water insoluble fractions were separated from the solution of water-soluble polysaccharides by centrifugation at 4000 rpm for 5 min. Water soluble extra polysaccharides were dialyzed, concentrated to 10 mL under reduced pressure (50°C), and further fractionated and purified by column chromatography.

Purification by dialysis

For dialysis, the exopolysaccharide solution was transferred to a length of dialysis tubing. The tubing was sufficiently long to accommodate the volume of the solution, to allow for a possible increase in volume during dialysis, and to permit tight closure at both ends. The filled tubing was then submerged in the dialysis buffer (distilled water) contained in a beaker that is placed and kept on magnetic stirrer (Remi 2MLH). Contact of the dialysis tubing with the

magnetic stirring bar was avoided, as this can easily damage the tubing. The dialysis buffer was replaced several times until solution of exopolysaccharide in tubing and outer was in the same condition.

Purification by column chromatography

DEAE-cellulose (anion exchanger) was purchased from Sigma (D0909) and this dry resin was regenerated for running the column chromatography by slightly modifying the method of Sigma Company product information and also from web information online (http://www.champa.kku.ac.th/katekaew).

Preparation of DEAE-cellulose resin

Five grams of DEAE-cellulose was slowly added to 0.1M sodium hydroxide (300 mL) with gentle stir for 30 min (pH reached to 13). Discarded the sodium hydroxide solution and washed the resin with double distilled water until pH reached to 8.0. Then solution was replaced with 0.1 M hydrochloric acid with gentle stir for 30 min. (pH reached to 1.0). Washed the resin until pH reached to 3.0 with double distilled water. Discarded the distilled water and replaced it with 10X buffer (500 mM tris HCl 8.0) and gentle stirrer for 30 min. Discarded the 10X buffer and then equilibrated the resin with 50 mM tris HCl 8.0, degassed and the fines removed before the suspension of DEAE-cellulose resin was transferred into a glass column.

DEAE-cellulose column chromatography

DEAE-cellulose powder was swollen when pretreated. The resin was packed into a column (Borosil 1x35 cm) to reach approximately 23 cm high, and then equilibrated with 50 mM tris-HCl, pH 8.0 with flow rate of 0.7-0.8 mL/min. Ten mL of dialyzed solution was applied to the DEAE-cellulose column and elution was performed with 50 mL of distilled water and followed with 50 mL of each 0.1 M phosphate buffer, 0.1 M NaHCO$_3$, 0.3 M NaHCO$_3$, 0.5 M NaHCO$_3$, 0.1 M phosphate buffer containing 1 M NaCl. The column effluents were collected with fractionation, 3 mL per fraction with a flow rate of 0.5 mL/min.

All fractions were estimated for total sugars by [4] using spectrophotometer (Elico SL159). From elution pattern, the absorption peaks were noted and fractions were stored for further use.

Characterization of exopolysaccharides

Characterization of exopolysaccharide was done using the standard methods [5, 6]. Exopolysaccharide was dissolved in DMSO and chemical shifts were expressed in ppm for H1 and C13 NMR [13]. C NMR spectra were acquired at 75 MHz and 125 MHz with TMS as internal standard for solutions in CDCl$_3$. J values were given in Hz. The positions of glycosidic linkages could be analyzed by NMR spectroscopy. GC-MS determination was carried out using ISL/CSM/5003. The polysaccharide was also characterized using a Fourier transform infrared spectroscopy on Perkin-Elmer infrared-683 spectrophotometer with KBr optics. All spectra were collected in the range of (4000-600 cm^{-1}). Proton magnetic spectra were recorded on Varian Gemini-200, Varian unity-400 and Varian FT-80A. All samples were made in DMSO-d6 using tetramethylsilane (Me$_4$Si) as the internal standard and are given in the δ scale. Biuret method was done for this isolated exopolysaccharide to confirm the presence of proteopolysaccharide. Schematic diagram of production, isolation, purification and characterization of exopolysaccharide from *Fomitopsis feei* was given in Fig. 1.

Fig-1: Schematic diagram of production, isolation, purification and characterization of exopolysaccharide from *Fomitopsis feei*

DEAE – cellulose anion-exchange chromatography column was used to isolate negatively charged polysaccharides from crude polysaccharides [7]. The precipitate was fractionated on a DEAE–cellulose column with different buffer solutions. There was absorption at 280 nm indicating peptide chain. Fractions (65-77) eluted at 198-236 mL were collected, dialyzed, concentrated and used for characterization. The elution profile on DEAE cellulose column is shown in Fig.2. Purification is important for getting polysaccharide of interest for many applications. The numbers indicate the fractions pooled together because they belong to a single peak.
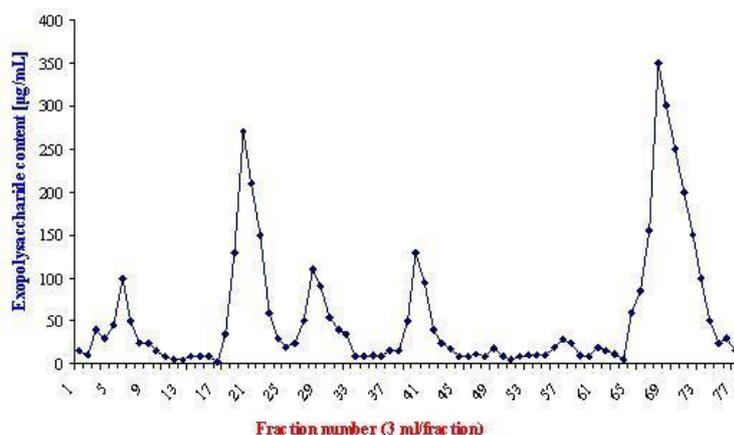

Fig-2: Elution profile of exopolysaccharides on DEAE cellulose column chromatography

Table-1: Effect of different production media on final pH, mycelial biomass and exopolysaccharide production of *Fomitopsis feei*

Type of production media	DOI	Initial pH	Final pH		Mycelial biomass (g/L)		Exopoly-saccharide(g/L)	
			Still	Shake	Still	Shake	Still	Shake
1	7	7.5	4.5	5.5	1.8	6.0	5.0	5.0
	14	7.5	2.0	0.5	6.0	9.8	3.3	3.3
2	7	4.0	4.5	5.5	1.4	1.0	-	-
	14	4.0	0.1	1.5	1.0	7.2	3.3	-
3	7	5.5	0.5	5.5	2.4	2.6	3.0	3.3
	14	5.5	1.0	0.8	4.8	6.2	3.3	-
4	7	5.5	2.0	4.0	4.8	1.6	2.0	-
	14	5.5	1.0	5.5	5.2	3.4	-	-
5	7	5.5	0.5	5.5	3.8	3.8	-	2.0
	14	5.5	0.5	0.5	5.8	6.4	3.3	3.3
6	7	5.5	1.0	5.5	3.8	3.2	5.0	2.0
	14	5.5	1.0	0.5	6.0	6.4	4.6	-
7	7	5.5	1.0	4.5	3.4	2.2	4.0	3.3
	14	5.5	1.0	1.0	6.2	4.8	-	3.3
8	7	6.5	1.5	5.5	4.2	1.4	5.0	3.3
	14	6.5	1.0	5.0	5.0	4.2	-	3.3
9	7	7.0	1.6	2.5	6.8	6.3	5.5	2.5
	14	7.0	1.7	1.9	6.0	10	4.0	4.0

DOI = Days of incubation; - = No production; 1 = Beta glucan production medium; 2 = GPCS medium (Glucose, polypeptone, canesugar, soybean oil); 3 = Fermentation medium; 4 = YMPG (Yeast extract, malt extract, peptone, glucose); 5 = SGP medium (Sucrose, glucose, peptone); 6 = Fungal growth medium; 7 = Standard polysaccharide production medium; 8 = Mushroom complete medium and 9 = Common production medium.

RESULTS AND DISCUSSION
Production of exopolysaccharides in different media

Nine types of production media viz. Beta glucan production medium [8], GPCS medium [9], Fermentation medium [10], YMPG [11], SGP medium [12], Fungal growth medium [13], Standard polysaccharide production medium [14], Mushroom complete medium [15] and Common production medium [16] were used for testing the production of exopolysaccharides. The effect of different media on the production of exopolysaccharide from *F.feei* is shown in Table 1.

Microbial exopolysaccharide production is usually considered as a metabolic strategy for survival and growth under unfavorable environmental conditions [17]. Usually, secondary metabolites are produced at the beginning of the deceleration phase (idiophase) [18]. The results of EPS production on various media was in the order of Common production medium > Beta glucan production medium > Fungal growth medium > Mushroom complete medium, based on production of exopolysaccharides both in still and shake cultures after 7 and 14 days of incubation. The initial pH of the fermentation media slowly decreased at the end of fermentation. There were no notable morphological changes in mycelium amongst the different culture media.

The exopolysaccharide production yields in YMPG medium were relatively lower than those in MCM medium. This result was in similarity with the earlier research report [19]. The yeast malt extract medium (YMPG) was shown to yield the highest production of EPS from *Coriolus versicolor* [20] which is not supported by our study since different species prefer different production media. Although fungal growth medium (6) given the highest exopolysaccharide production in still culture, showed very less in shake condition both after 7 and 14 days. Good mycelial growth seems not to be a determining factor for the high production of exopolysaccharides in *F.feei*. This result is in agreement with the results of earlier research for *Ganoderma lucidum* [1] and *Phellinus linteus* [19]. Comparing the EPS production on the 9 types of media, Common production medium (9) supported the best production of exopolysaccharides in both shake and still conditions after 7 and 14 days of incubation (Fig. 3). Hence, this medium was selected for further production and optimization of exopolysaccharides from *Fomitopsis feei*.
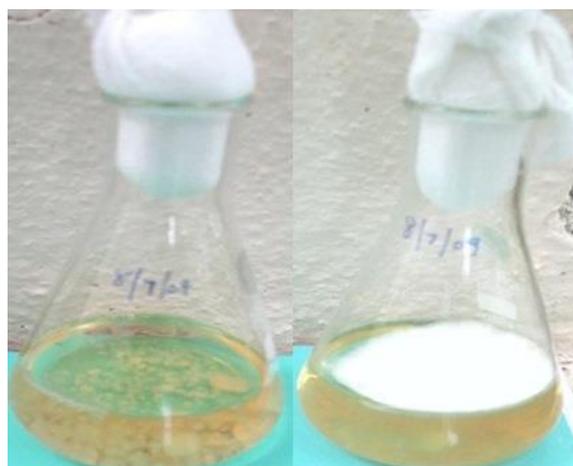

Fig-3: Growth of *Fomitopsis feei* in shake and still conditions

Earlier research [22] disclosed that solvent used for precipitation was four times to the filtrate so that total exopolysaccarides were precipitated without losing. Isopropanol was found to be good for the best precipitation of polysaccharides [23]. Hence for the excellent precipitation of exopolysaccharides, 1:4 ratio of solvent was used in both still and shake conditions after 7 and 14 days of incubation.

It is difficult to compare the chemical composition in the present study with those of other reports, since most of previous data in the literature are obtained from extracts of fruit bodies or mycelia but not of exopolysaccharide from culture filtrates. All the peaks were compared with the literature values. The β-configuration was shown by H-1 signals at high field and C-1 signals at low field. C13 and H1 NMR determinations were carried out and samples were dissolved in DMSO. Chemical shifts are expressed in ppm.

From the spectral analysis of exopolysaccharide of *Fomitopsis feei,* monomer is predicted as glucose (3.64 ppm). Mass spectrometry is analytical technique for the determination of the elemental composition of a sample or molecule. The infrared spectroscopy has become a standard method for the analysis of extracted fungal polysaccharide [24,25,26]. 1000-1500 cm^{-1} IR region is really a

carbohydrate "fingerprint" region by which each class of polysaccharides can be recognized [27].

Furthermore, bands around 890 cm^{-1}, 900 cm^{-1} and 1370 cm^{-1} are revealed to β configuration of the glucan linkages. In addition, band around 1150 cm^{-1} is due to glucose (1,4) – di – O – substituted. These assignments agree with those presented by other authors for other types of polysaccharides [25,28]. The FTIR spectra suggested that the presence of a small amount of protein band at (1540 cm^{-1}) which was confirmed by the Biuret method and the presence of uronic acids (carbonyl bands over 1700 cm^{-1}). The characteristic bands of beta glucans occurred in the 1000-1100 cm^{-1} region due to the O-substituted glucose residues. The band at 1410 cm^{-1} evidenced the presence of a beta glucan. These findings also supported the previous study [29].

Furthermore, a continuous absorption at approximately 3400 cm^{1} is characteristic of a carbohydrate ring [30]. It was concluded that no α-configuration exists since there was no characteristic absorption band at 840 cm^{-1} based on previous research report [31]. The band at 1150 cm^{-1} was due to (1,3)-di-O-substituted glucose residue [32]. The absorptions at 1075, 1219 and 1269 cm^{-1} indicated a pyranose form of glycosyl residue in earlier report [33-35].

The data obtained from C13 NMR (Fig. 4), H1 NMR (Fig. 5), GC-MS (Fig. 6), FTIR (Fig. 7) and by manual analysis it is confirmed that this is a proteopolysaccharide (beta glucan).
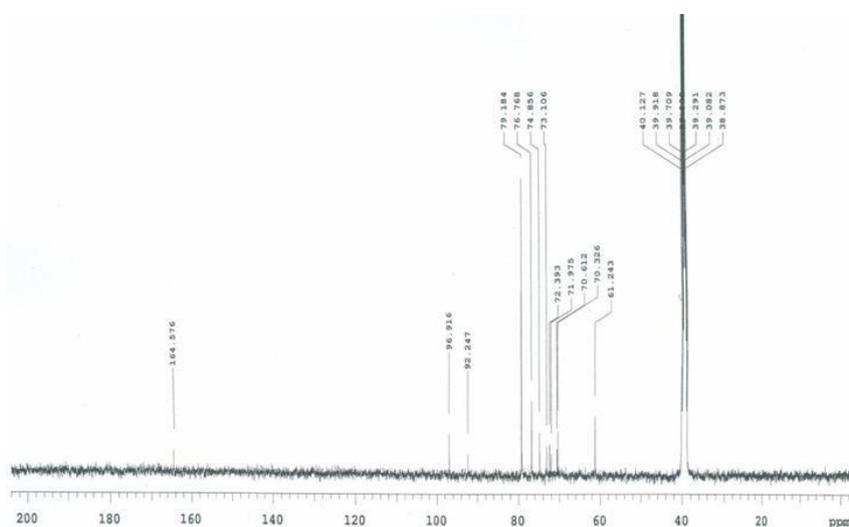

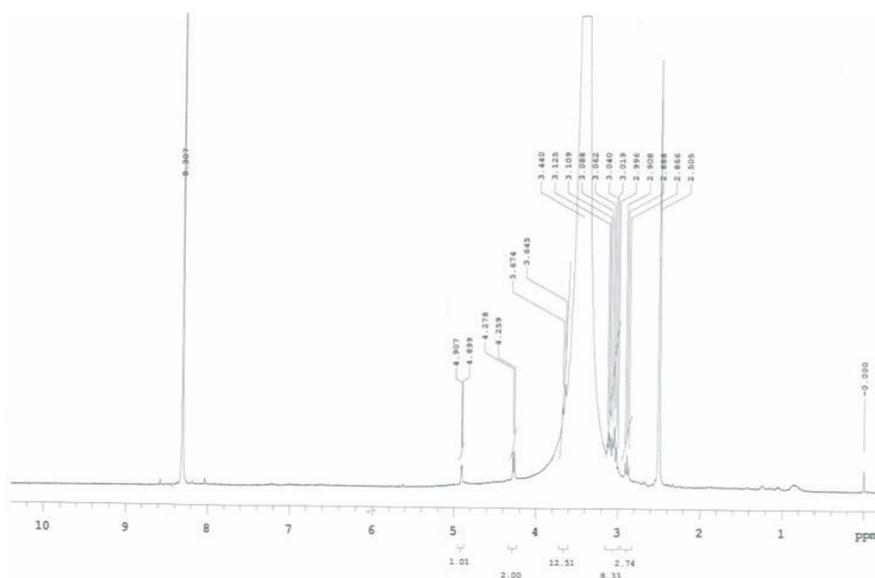**Fig-4: C13 Nuclear Magnetic Resonance Spectroscopy**

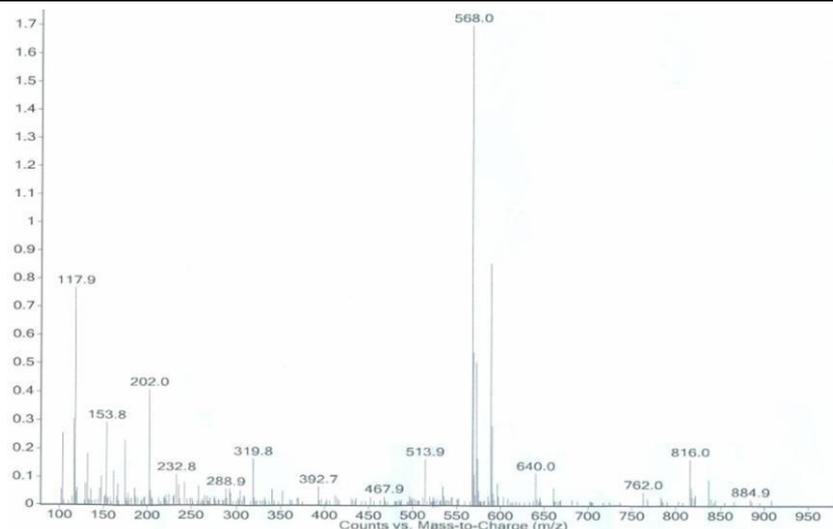**Fig-5: H1 Nuclear Magnetic Resonance Spectroscopy**

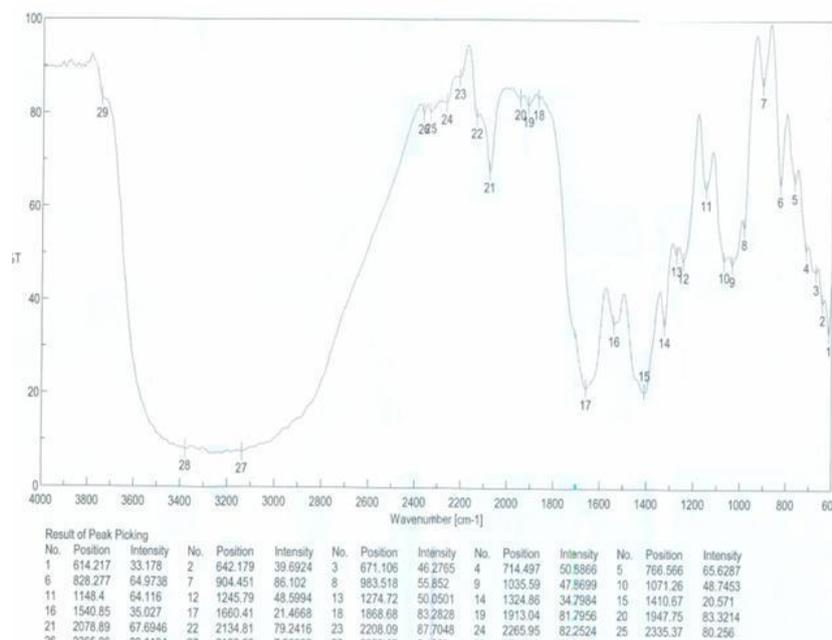**Fig-6: Gas Chromatography and Mass Spectrometry**

Fig-7: Fourier Transform Infra-Red Spectroscopy

CONCLUSIONS

Crude polysaccharide was purified using DEAE column chromatography but it was difficult to compare the spectral data in the present study with those of other reports, since most of previous data in the literature are obtained from extracts of fruit bodies or mycelia not of EPS. Finally, comparing with the characterization result with previous literature it is concluded that it is a proteopolysaccharide.

From the aforementioned results, a critical conclusion was derived that isolation of active ingredients from *Fomitopsis feei* with mechanism based potential therapeutic value still requires intensive investigation, especially with the emergence of new evidence of their health benefit effects. Although our research has concluded some vital results further investigations are needed to clarify the mechanisms.

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