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Isolation and characterization of an extracellular polysaccharide from *Pseudomonas caryophylli* CFR 1705

S.R. Sudhamani[a], R.N. Tharanathan[b,*], M.S. Prasad[a]

[a]*Department of Food Microbiology, Central Food Technological Research Institute, Mysore 570 013, India*
[b]*Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore 570 013, India*

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Abstract

Extracellular polysaccharides were isolated from *Pseudomonas caryophylli* CFR 1705 grown on lactose containing medium. The major fraction (no.1) obtained on DEAE-cellulose chromatography was composed of rhamnose, mannose and glucose in the ratio 1:3.26:4.97, respectively, and having a molecular weight of 1.1×10^6 Da. Methylation followed by GC-MS analysis revealed it to be a highly branched 1,4-linked hexosan with mannose and glucose as the branch-off residues at positions C-2 and C-6 of the main chain. Rhamnose was essentially found as non-reducing terminal residue.

Keywords: Extracellular polysaccharide; *Pseudomonas caryophylli*; Purification; Characterization; Structure

1. Introduction

Polysaccharides are incorporated into foods as thickening, suspending or gelling agents in order to improve food quality and texture (Stephen, 1995). Industrial use of polysaccharides has until recently relied to a considerable extent on materials from plants and marine algae and typical examples include starch, pectin, alginate, carrageenan, galactomannans and a variety of exudates and seed gums (Tharanathan, 1995). Bacterial polysaccharides offer a viable alternative to the currently used traditional polysaccharides and find extensive application in food and pharmaceutical industries (Sutherland, 1983).

The unique rheological properties of xanthan have generated new markets for its use as a thickening, suspending and gelling agent (Roller & Dea, 1992). Gellan gum is a relatively new bacterial gum, which has food approval in Japan and USA. Curdlan, Pullulan, Alginates, Scleroglucan are some of the important microbial polysaccharides which are finding application in different areas of industry. The advantages of bacterial polysaccharides are novel functionality, constant and reproducible chemical and physical properties and a stable cost and supply (Maccormick et al., 1996). There is a growing interest in the isolation of newer polysaccharides with better rheological properties and varied application potential. This article describes the isolation and preliminary characterization of one such polysaccharide from *Pseudomonas caryophylli*.

2. Materials and methods

2.1. Isolation of polysaccharide producing organism

Mucoid bacterial colony was isolated from soil sample and was maintained on a lactose-agar medium at 30 °C. The organism has been identified as *Ps. caryophylli* based on morphological and biochemical tests according to the Bergey's manual of Determinative Bacteriology (Holt, Krieg, Sneath, Stealey, & Williams, 1994). The strain was deposited in the culture collection center of CFTRI, and labeled as CFR 1705.

2.2. Polysaccharide isolation and purification

Ps. caryophylli CFR 1705 was grown aerobically for 72 h in a lactose-agar medium at 30 °C and 200 rpm on a rotary shaker (Remi Instruments, Bombay, India).

* Corresponding author. Tel.: +91-821-251-4876; fax: +91-821-251-7233.
E-mail address: tharanathan@yahoo.co.uk (R.N. Tharanathan).

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The viscous broth was pasteurized for 15 min at 70 °C and centrifuged at 15,000*g* for 20 min. The supernatant fluid was removed and the cells were washed with distilled water and recentrifuged. The exopolysaccharide (EPS) was precipitated by adding two volumes of chilled isopropanol to the supernatant fluid. The resultant polysaccharide (EPS) was collected by centrifugation and its aqueous solution was dialyzed against distilled water at 4 °C for 36 h and lyophilized.

2.3. Analytical methods

All chemicals and reagents were obtained from Sigma Chemicals Co., St Louis, MO, USA, unless otherwise stated. Total carbohydrate content was determined by the method of McKelvy and Lee (1969).

2.4. Physiological tests

2.4.1. Effect of carbon source on polysaccharide production

The bacterial isolate was incubated at 30 °C for 72 h in the medium containing different carbon sources such as sucrose, lactose, maltose, glucose, galactose, fructose and xylose, and later EPS isolated.

2.4.2. Effect of pH on polysaccharide production

The bacterial isolate was incubated at 30 °C for 72 h in lactose broth medium, whose pH was adjusted to 3, 4, 5, 6, 7, 8, 9 and 10 using either dil. HCl or dil. NaOH, and EPS isolated.

2.4.3. Effect of temperature on polysaccharide production

The bacterial isolate was incubated for 72 h in lactose broth medium (pH 7.5) at 12, 16, 20, 24, 28, 32, 36 and 40 °C, and EPS isolated.

2.5. Ion exchange chromatography

Lyophilized EPS (100 mg in 5 ml water) was subjected to anion exchange chromatography on a column (2.5 × 22 cm) of DEAE-cellulose, pre-equilibrated using 0.5 M ammonium carbonate. The column was washed with deionised water followed by successive elutions with ammonium carbonate solution of increasing molarities (0.1,0.2 and 0.3 M) in a stepwise manner followed by elutions with 0.1 and 0.2 N NaOH. Fractions (10 ml) were collected and an aliquot (0.5 ml) was tested for total carbohydrates. The respective polysaccharide fractions were pooled and dialyzed overnight against distilled water and lyophilized.

2.6. Gel permeation chromatography

Lyophilized 0.2 M ammonium carbonate and 0.2 M NaOH eluted fractions were subjected to gel permeation chromatography on a column (1.2 × 132 cm) of Sephacryl S-400 (Pharmacia, Sweden), which was pre-calibrated with T-series Dextrans (Pharmacia, Sweden, of known molecular weights). The void volume was determined using blue dextran. The column was equilibrated and later eluted with 0.1 M NaCl. Aqueous solution of sample (10 mg) was dissolved in 1.5 ml of 0.1 M NaCl and loaded on to the column bed. Fractions (3 ml) were collected and tested for total carbohydrates.

2.7. Gas liquid chromatography

Sample (10 mg) was taken in TFA (2 M, 1 ml) and the tube was heat-sealed. Hydrolysis was carried out at 100 °C for 4 h in an oven. After the hydrolysis, the acid was removed by flash evaporation at a water bath temperature of 40 °C and co-distilled with water (1 ml × 3). To the hydrolyzed sample in water (0.5 ml) sodium borohydride (20 mg) was added and left overnight. Excess borohydride was destroyed by the addition of dilute acetic acid (2 M) and the borate formed was removed by co-distilling with methanol (1 ml × 4) to dryness. Dry and distilled acetic anhydride and pyridine (0.5 ml each) were added and kept in an oven at 100 °C for 2 h. Excess reagents were removed by co-distilling with water (1 ml × 3) and toluene (1 ml × 3). After thorough drying, the contents were taken in chloroform for gas liquid chromatography (GLC) analysis (Sawardekar, Slonekar, & Jeanes, 1967).

2.8. Methylation of polysaccharide

Methylation of the polysaccharide was done as described by the Hakomori (1964) method. Since Fraction II could not be completely solubilised in DMSO, only Fraction I was taken for the analysis. Polysaccharide (5 mg) was dissolved in dry distilled DMSO (0.5 ml) with stirring and occasional ultrasonication. Methylsulphinyl carbanion (1 ml) was added to the above solution and the mixture was stirred at room temperature for 3–4 h. After the reaction, the mixture was tested for excess anion content by reacting with triphenylmethane with which it gave a blood red colouration (Rauvala, 1979). Iodomethane (1 ml) was added to the reaction mixture at ice-cold temperature with the help of a syringe and left stirring for 2 h.

The methylated polysaccharide was purified in a Sep-Pak C18 cartridge. The methylated reaction mixture was diluted with equal volume of water and was passed through the cartridge. More polar contaminants of the reaction such as DMSO and sodium iodide were eluted with water (2 ml × 4), whereas less polar contaminants held on the cartridge were eluted successively with 2 ml each of the following: acetonitrile–water 3:17 (v/v, × 4), acetonitrile–water 1:4 (v/v, × 4), 100% acetonitrile (× 4), 100% methanol (× 4) and 95% methanol (× 4). The cartridge was flushed with these solvent mixtures at a rate of about 1–2 drops/s. The methylated sample was eluted in 100% acetonitrile and 100% methanol.

Table 1
Physiological and biochemical characteristics of the bacterial isolate CFR 1705

Characteristics	CFR 1705
Catalase	Positive
Oxidative	Fairly positive
Arginine	Positive
Starch hydrolysis	Negative
Growth at 41 °C	Positive
Growth at 4 °C	Negative
Gelatin hydrolysis	Negative
Lecithinase activity	Negative
Polyhydroxy butyrate	Present
Pigmentation	Nil

The permethylated polysaccharide was hydrolyzed in 2 ml formic acid (90%) at 100 °C for 2 h in a boiling water bath. The excess acid was evaporated by co-distillation with methanol (1 ml × 4). It was then re-hydrolyzed by TFA (2 M, 1 ml) in a sealed tube at 100 °C in an oven for 4 h. The acid was removed by co-distilling with water and the liberated sugars were reduced using sodium borodeuteride in D_2O and then acetylated, as before.

2.9. GLC-MS analysis

GLC-MS analysis was carried out on Shimadzu-QP5000 instrument using a SP-2380 fused silica capillary column (30 m, i.d. 0.32 mm, film thickness 0.02 μ, Supelco, USA). A temperature programming of 180–200 °C with an increase of 4 °C/min was maintained for the analysis. Ionization potential was 70 eV and mass range (m/Z) was 40–400 amu. Helium was the carrier gas used.

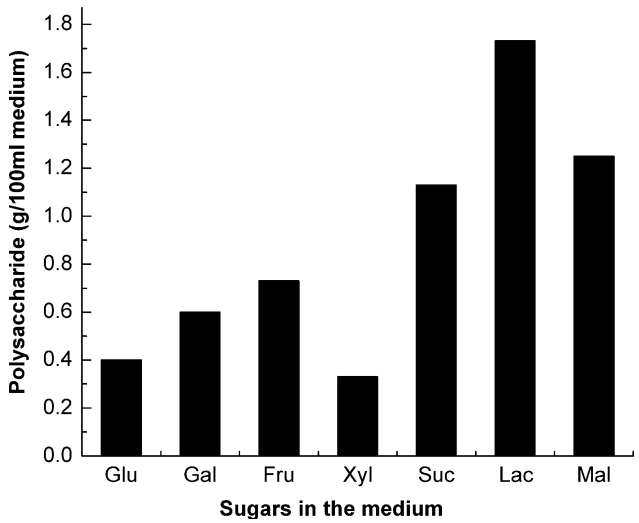

Fig. 1. Effect of sugars on EPS production by *Ps. caryophylli* CFR1705 (Glc, glucose; Gal, galactose; Fru, fructose; Xyl, xylose; Suc, sucrose; Lac, lactose; Mal, maltose).

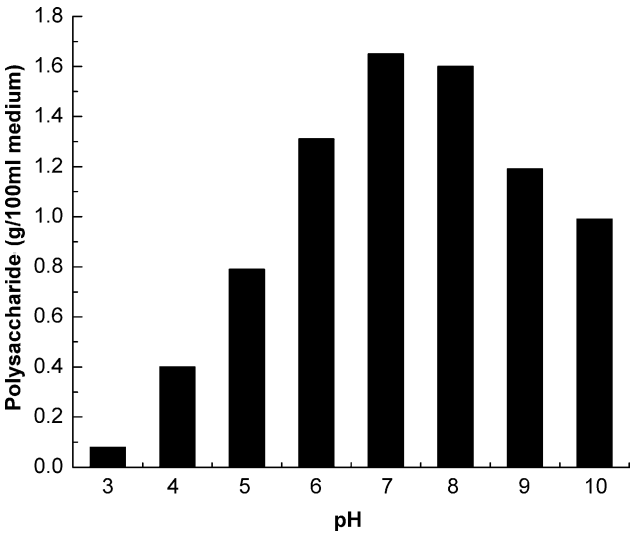

Fig. 2. Effect of pH on EPS production by *Ps. caryophylli* CFR1705.

3. Results

3.1. Characteristics of EPS producing organism

Colonies of *Ps. caryophylli* CFR1705 showed mucoid appearance on lactose-agar medium. The bacterial cells were Gram-negative, coccobacilli, whose biochemical characteristics are summarized in Table 1. Lactose was preferentially utilized by the organism for the maximum production of EPS compared to other carbon sources used (Fig. 1). The maximum EPS production was observed over a range of pH 7–8 (Fig. 2) and temperature 20–24 °C (Fig. 3).

3.2. Chemical characterization of crude EPS

The crude EPS was resolved by anion exchange chromatography on DEAE-cellulose into two major

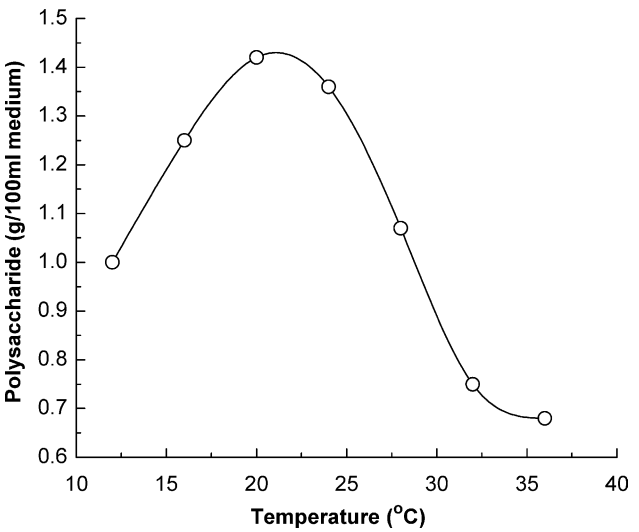

Fig. 3. Effect of temperature on EPS production by *Ps. caryophylli* CFR1705.

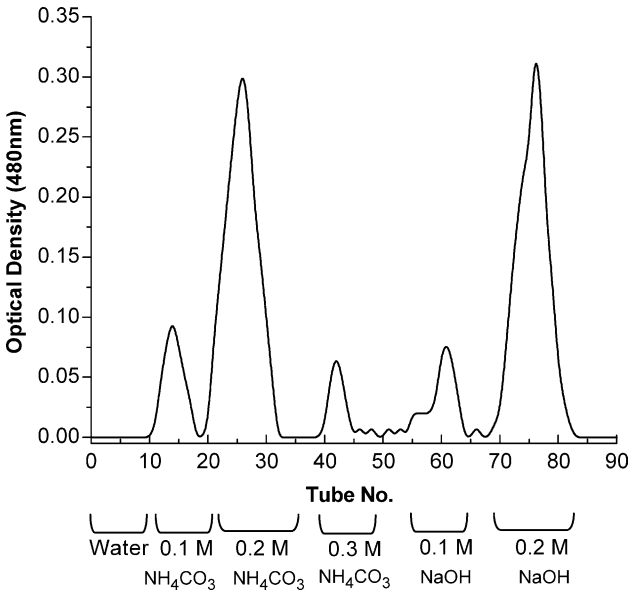

Fig. 4. Anion exchange chromatography of EPS from *Ps. caryophylli* CFR 1705 on DEAE-cellulose.

fractions, eluted with 0.2 M ammonium carbonate (Fra. I) and 0.2 M NaOH (Fra. II) in 33 and 36% yields, respectively, along with three minor fractions (Fig. 4). Analysis of their acid hydrolysates by GLC indicated the presence of rhamnose, mannose and glucose in the ratio of 1:3.26:4.97 in Fra. I and rhamnose, arabinose, xylose, mannose and glucose in the ratio of 1:0.31:0.26:3.54:3.82 in Fra. II, respectively. An estimation of their molecular weight by GPC showed values of about 1.1×10^6 and 1.2×10^6 Da, respectively, for Fras. I and II. Appearance of a single symmetrical peak in GPC profiles indicated their apparent homogeneity.

The results of methylation analysis of Fra. I are shown in Table 2. Identification of larger amounts of 2,3,6- tri-*O*-methyl derivatives of mannose and glucose in relatively comparable concentration showed pre-ponderance of 1,4-glycosidic linkages in the polymer backbone. Identification of 3,6-di-*O*-methylmannose and 2,3-di-*O*-methylglucose revealed considerable amount of branching at positions C-2 and C-6 of the main chain mannose and glucose residues, respectively. Rhamnose was essentially found as non-reducing terminal residue as indicated by the presence of 2,3,4-tri-*O*-methylrhamnose.

4. Discussion

There are many reports on the production of EPSs from *Pseudomonas* species and to our knowledge, this is the first report on the production of EPS from *Ps. caryophylli.* However, De Castro, Lanzetta, Molinaro, Parilli, and Piscopo (2001) have isolated an *O*-specific polysaccharide from the lipopolysaccharide (LPS) of *Ps.* (*Burkholderia*) *caryophylli*. The LPS was isolated from the dry cells by extraction with phenol–water mixture. In the present study the extracellular polysaccharide elaborated by the organism *Ps. caryophylli* was isolated by isopropanol precipitation. The isolated *Ps. caryophylli* produced the highest amount of EPS when lactose was used as the primary carbon source in liquid media (1.7 g of crude polymer/100 ml of nutrient medium). This represented an approximate 100–300% increase in yield over EPS production in media containing sucrose, maltose, glucose, galactose, fructose and xylose. Preliminary trials on whey (a by-product of diary industry) indicated the utilization of a cheaper carbon source for EPS production. The initial pH of the culture medium had little effect on EPS production, acidic pH did not support the production of EPS.

Fractionation of EPS on DEAE-cellulose indicated the presence of two major fractions (Fras. I and II) in the crude EPS. Sugar analysis of the fractions showed rhamnose, mannose and glucose. Traces of xylose and arabinose were observed in Fra. II. Presence of similar type of sugars was observed in the extracellular polysaccharide, Whelan from *Alcaligenes* ATCC 31555. However, the relative ratio of

Table 2
Partially methylated alditol acetate derivatives from permethylated Fra. I

Sugar derivatives	Absolute Rt (min)	Rt[a]	Relative proportion (%)	Mass spectral fragments m/Z	Mode of linkage
2,3,4-Me_3-Rha	4.49	0.61	12.16	43, 89, 102, 118, 131, 143, 162, 175	Rha (1 →
2,3,4,6-Me_4-Glc	7.28	1.00	1.51	43, 45, 101, 102, 118, 129, 161, 162, 205	Glc-(1 →
2,3,6-Me_3-Man	10.67	1.45	18.42	43, 45, 87, 102, 118, 129, 143, 161, 173, 203, 233	→ 4)-Man-(1 →
2,3,6-Me_3-Glc	10.87	1.49	22.34	43, 45, 87, 101, 118, 129, 143, 161, 178, 203, 233	→ 4)-Glc-(1 →
3,6-Me_2-Man	18.11	2.49	21.19	43, 45, 87, 98, 115, 129, 130, 190, 233	→ 2,4)-Man-(1 →
2,3-Me_2-Glc	21.44	2.94	20.69	43, 85, 99, 102, 118, 129, 162, 189, 201, 261, 315	→ 4,6)-Glc-(1 →

[a] With respect to 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-D-glucitol.

the sugars was entirely different with rhamnose, mannose and glucose in 46:11:43, respectively (Jansson, Lindberg, & Widmaim,1985). The sugar composition of heteropolysaccharides produced by bacteria would generally include galactose, mannose, glucose and rhamnose.

Permethylation analysis is an excellent method to determine the glycosidic linkages as well as to find out the linearity or otherwise of the polymeric molecule involved in the backbone. Methylation studies have been carried out to elucidate the structural linkages in xanthan, gellan and curdlan (Jansson & Lindberg, 1983; Jansson et al., 1985; Lindberg, Lonngren, & Thompson, 1973). From the results of GC-MS analysis of the permethylated Fra. I, it may be deduced that the polysaccharide is a highly branched hexosan with mannose and glucose as the branch-off residues. A fairly good agreement in the relative proportion of the constituent sugars before and after methylation lends support for the structural features deduced for this polysaccharide.

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