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EXOPOLYSACCHARIDE PRODUCTION BY INDIGENOUS SOIL BACTERIUM *PSEUDOMONAS STUTZERI* CMG1436

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ABSTRACT

Bacterial strain CMG 1436 identified as *Pseudomonas stutzeri* was found to produce an extracellular anionic heteropolysaccharide in growth medium. By acid hydrolysis exopolysaccharide was depolymerized into glucuronic acid, D-glucose and D-arabinose monomers. Their separation on Whattman paper No. 3 revealed that the number of D-glucose and D-arabinose monomers was found to be concentrated whereas that of glucuronic acid was lower. Further, like other bacterial polysaccharides, this exopolysaccharide was found to be high molecular weight polymer and its molecular weight was estimated in the range >20,000 and <250,000 Dalton.

Keywords: Exopolysaccharide, electron microscopy, acid hydrolysis, relative molecular weight.

INTRODUCTION

Bacteria are known as potential source to produce incredibly diverse range of exopolysaccharides (EPSs) with equal immense importance and potential applications in food and non food industries. These are either secreted in the growth medium or remain attached to the bacterial cell wall as viscous, ropy or slime layers in the growth medium (Robbert, 1995). Most, but not all of the functions ascribed to EPS are of immunogenic, survival and protective for bacteria living within varied natural environment against desiccation, starvation, stressful conditions, phagocytosis and predation (Whitfield, 1988). Based on their monosaccharide composition, bacterial EPSs have been grouped into homopolysaccharides and heteropolysaccharides. Homopolysaccharides are common in gram positive bacteria synthesized outside the cell solely by the action of bacterial extracellular enzymes such as dextrans and levan (Yang, 2000). Whereas heteropolysaccharides are synthesized within the cell and then excreted past the bacterial outer membrane or cell wall. This system of EPS biosynthesis is a complex mechanism and has been well-defined in gram-negative bacteria (Broadbent *et al*., 2003). Bacterial EPSs including most of the heteropolysaccharides are usually acidic (anionic) in nature because they carry a negative charge that can be imparted by the presence of uronic acids or non-carbohydrate substituents such as pyruvate, sialic acid, succinate, lactic acid, phosphate and carboxylate groups such as xanthan, alginate and succinoglycan etc (Fett, 2001). Whereas the majority of homopolysaccharides are neutral e.g. cellulose, dextran and levan etc. To date, at least 20 different monosaccharides have been identified in EPSs produced by a variety of bacteria. Of these, heteropolysaccharides are very common than homopolysaccharides and are more diverse because of copolymerization of various monomer units results in various possible types of linkage, various repeats of monomers in chain and their relative ratio of combination (Kenne and Lindberg, 1983). This diversity arises due to environmental and bacterial growth conditions and also depends on bacteria themselves (Tina *et al*., 2001). Because of these variations, bacterial EPSs do not have an exact molecular weight and it is distributed over a broad range typically in10^3-10^6 (Sutherland, 1982 & 1985). Over the years, extensive studies on EPS production have focused mainly on bacteria from marine, water, fruits, plant and clinical sources (Whitefield, 1988), whereas relatively little attention has been paid to EPS production by soil bacteria. Therefore, the present study was aimed at the production and characterization of EPS by indigenous soil bacterial strain CMG1436.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Gram negative soil bacterium CMG 1436 was identified by using DESTO QTS-24. Minimal medium for the production of exopolysaccharide (EPS) was prepared from the following constituents; 2% sucrose as carbon (separately autoclaved at 110^{o}C for 15 minutes), 0.68% KH_2PO_4, 0.88% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.01% NaCl, 0.05% Yeast Extract, 0.05% Urea (filtered by millipore filter, 0.22 µm). The medium was adjusted to pH 7. Bacterial strain CMG1436 was grown over night in above medium at 30^{o}C. one ml of seed culture was inoculated into 2000ml flask containing 1litre above medium, and incubated at 30^{o}C for 5 days.

Transmission electron microscopy

Sample preparation was carried out according to the method as described by Madilyn and Floodgate, (1973). Ultrathin sections (60nm) were cut on an LKB ultramicrotome using glass knives, stained with lead citrate (Reynolds, 1963), and examined in JEOL transmission electron microscope JEM 1200 EX II operating at 60 KV.

Extraction of exopolysaccharide

five days old culture was centrifuged (12000 rpm) for 30 minutes at 4^{o}C to sediment the cells. Extracellular protein fractions were removed by the method of Raymond (1988) and viscous supernatant was added to two volumes of absolute ethanol. The resultant precipitates were collected and washed three times with distilled water. Precipitates were dried in wheaten dry-seal vacuum desiccator over $CaCl_2$ until a constant weight was reached and recorded as crude EPS. For further purification, EPS was subjected to dialysis in dialysis tubing (diameter 16mm) with a cut off value of M_r12,400 Dalton or greater against 3litre distilled water for 72hrs with periodical (24hrs) change of distilled water. Dialyzed EPS was lyophilized at -50^{o}C with a constant vacuum of 70×10^{-3} MBAR using LABCONCO Freeze Dry System 77520 and recorded as pure EPS.

Measurement of moisture and ash content

Moisture, ash (inorganic) and organic contents of EPS was determined by the methods of Horwitz (2000) in triplicate and average values were calculated.

Quantitative analysis

Chemical composition determination was made in triplicate, and average values were estimated. uronic acid was determined by carbazol reaction (Bitter and Muir, 1962) with D-mannuronic acid standard, protein was estimated by Bradford method with bovine serum albumin standard (Bradford, 1978), total neutral sugar was determined by phenol-sulphuric acid method with D-glucose standard (Kochert, 1978) and total lipid content was estimated by the gravimetric method of Salton (1953).

Acid hydrolysis

One mg of EPS was dissolved in 500µl 2M trifluoro-acetic acid (TFA) into an airtight vial. For optimizing the hydrolyzing conditions EPS was hydrolyzed at 120^{o}C for 1.5, 3 and 5 hrs. Then the hydrolyzed sample was dried over night in a Wheaton dry-seal vacuum over $CaCl_2$ and then resuspended in 50µl of distilled water for paper chromatography (Georg and William. 1974).

Paper chromatography

Hydrolysate and monosaccharide standards (D-glucuronic, D-glucuronolactone, galacturonic acid, D-glucose, D-galactose, D-xylose, D-arabnose, D-mannose, L-fucose and L-rhamnose) were spotted on Whattman paper No. 3 and dried in air. Descending Paper Chromatography (DPC) was carried out for 18 hrs at room temperature with solvent system BAW consisting of BuOH: Acetic acid: water (4:1:5), shaken well and top layer was used. Paper was dried in air and stained with alkaline silver nitrate reagent (George and William, 1974).

Relative molecular weight determination

Thirty mg of EPS with known sugar content was dissolved in 3ml distilled water and applied on Sephadex G-50, column I (72× 1.5cm), eluted with distilled water at a flow rate of 0.5ml/minute at ambient. High molecular weight sugar fractions pooled from column I were filtered, lyophilized and rechromatographed on Sephadex G-100 column II (72×1.5cm) with same elution conditions. Sugar content of each fraction was determined by phenol-sulphuric acid assay with D-glucose as standard. Columns were calibrated with broad standard Dextran (T10, T20 and T250) for void volume (V_o) and with Glucose for included volume (V_i).

RESULTS AND DISCUSSION

Indigenous soil bacterium CMG1436 previously phenotypically characterized and found to produce an acidic EPS was now identified as *Pseudomonas stutzeri*. Its EPS production was examined by transmission electron microscopy of culture broth. Figure 1 showed that EPS present intracellularly, and extracellularly surrounding the cells has been negatively stained by Ruthenium Red. Therefore, it was suggested that EPS was acidic in nature which was initially synthesized intracellularly and then transported into the medium outside the cell. Ruthenium Red staining has been widely used in electron microscopy for the examination of bacterial acidic exopolysaccharides production (Corpe, 1970). After extraction, physically a homogenous and pure white polymer was obtained. Figure

2 showed that vacuumed-dried crude EPS contained an average content of moisture, organic matter and ash in 12.9%, 75.9% and 10.9% respectively. While those in pure were estimated as 16.5, 80.3 and 3.1% respectively. In purified EPS, moisture and organic contents were increased but that of ash was reduced than those in crude EPS. These results suggested that CMG1436 EPS was an organic and hygroscopic compound in nature. As for as the chemical composition pattern was concerned, both crude and purified EPS samples were rich in neutral sugar content (Table 1).

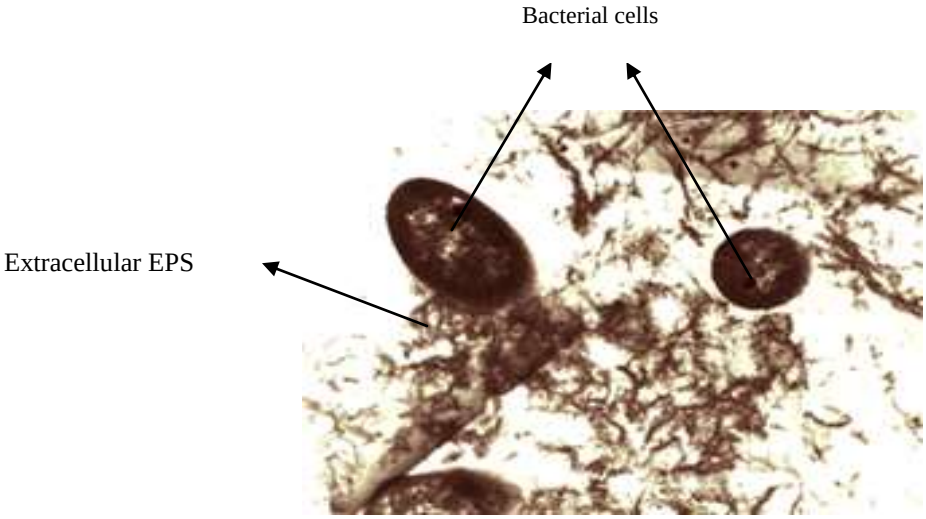

Fig 1. Transmission electron micrograph showing biosynthesis of EPS by *Pseudomonas stutzeri* CMG1436.

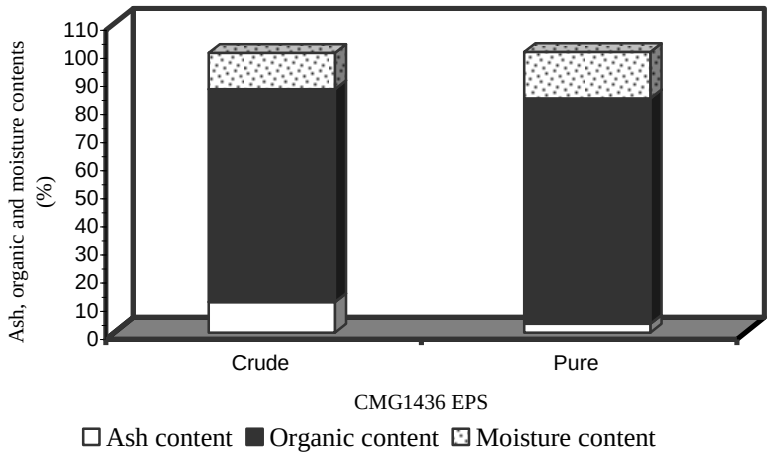

Fig 2. Distribution pattern of ash, organic and moisture contents in EPS produced by *Pseudomonas stutzeri*CMG1436.

Table 1. Chemical composition pattern of EPS

EPS	Content expressed as g% w/w				
	Neutral sugar	Protein	Uronic acid	Phophorus	Lipid
Crude	91.31	1.8	2.52	3.882	-
Pure	96.584	-	3.367	-	-

Table 2. Monomer composition of EPS released by TFA hydrolysis.

Standard monosaccharides	R_f values	Tentative assignment of hydrolysate spots released in different lengthof time		
		1.5hr	3hr	5hr
D-Galacturonic.acid	7.48			
D-Glucuronic.acid	10.59		+	+
D-Galactose	19.18			
D-Glucose	20.82	+	++	+
D-Mannose	24.67			
D-Arabinose	25.55			++
D-Xylose	29.73			
L-Fucose	35.46			
D-Glucuronolactone	43.65		+	+
L-Rhamnose	44.47			

+: Light spot, ++: Dark spot.

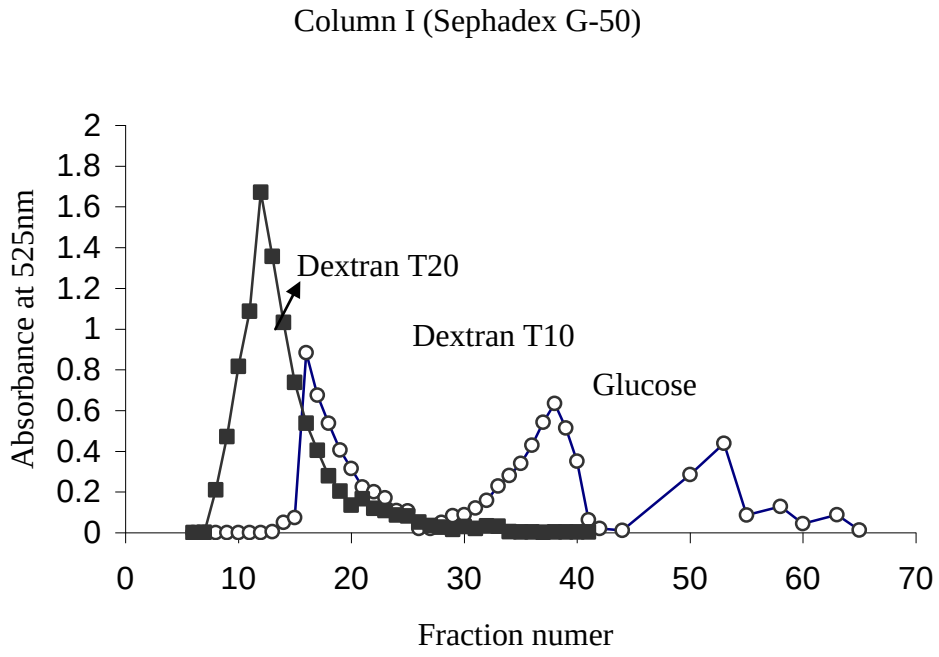

Fig. 3A. Gel permeation chromatography elution profile of EPS produced by *Pseudomonas stutzeri* CMG1436 (■) and molecular weight standards (○) on Sephadex G-50.

In composition pattern of crude EPS, a trace amount of protein, uronic acid and phosphorus were also detected. After purification, protein and phosphorus fractions were disappeared while content of neutral sugar and uronic acid

was comparatively increased (Table 1). Therefore, it has been suggested that this EPS most probably might be an acidic EPS. These results were further substantiated by acid hydrolysis of exopolysaccharide. As result of acid hydrolysis for 1.5 3 and 5hrs, four spots were developed on paper chromatogram whose R_f values were in agreement with glucuronic acid, D-glucose, D-arabinose and D-glucuronolactone (table 2). D-glucose and D-arabinose aligning spots released in 3 and 5hrs hydrolysis were concentrated as compared to other spots which were light. So, this paper chromatogram suggested that EPS produced by CMG1436 was an acidic heteropolysaccharide containing glucuronic acid with lower concentration while, D-glucose and D-arabinose with higher concentration in its backbone. As elution profile of ESP from Sephadex G-50 showed that most of the sample eluted before void volume, giving an apparent range of molecular weight, >20,000 Dalton (fig. 3A). However, a trace amount of EPS was also eluted within void volume (DextranT10), suggesting the low molecular weight EPS or oligosaccharides. The another possible reason for elution of sample within void volume might be that adhering to column gel due to their stereochemistry may leave behind some EPS molecules so could not elute with high molecular weight EPS and lagged behind. Similarly, separation profile achieved in Sephadex G-100, showed that whole of the applied EPS sample was eluted near after the void volume, suggesting a molecular weight, <250,000 Dalton (fig. 3B). Hence by gel permeation chromatography, a relative dispersion of molecular weight of EPS produced by *Pseudomonas stutzeri* CMG1436 was established in the range >20,000 and <250,000 Dalton. This range of molecular weight interestingly fell within the range of polysaccharide produced by up-to date reported bacterial strains (Whitefield, 1988).

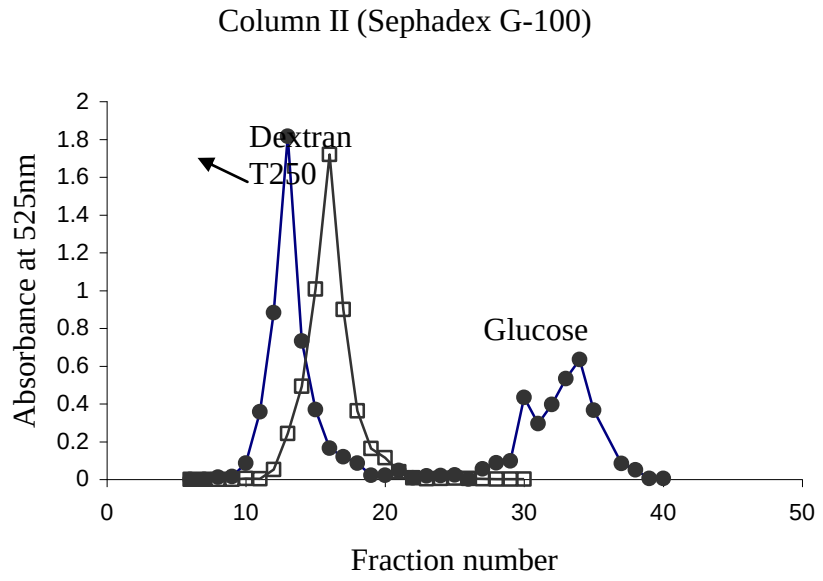

Fig. 3B. Gel permeation chromatography elution profile of EPS produced by *Pseudomonas stutzeri* CMG1436 (●) and molecular weight standards (□) on Sephadex G-100.

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