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Histochemical and Physicochemical Study of Bacterial Exopolysaccharides

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Abstract: Present study shows the complexity of determining the chemical composition of three exopolysaccharides obtained from bacteria associated with plant roots grown under salt stress conditions. Physicochemical analysis indicated that the exopolysaccharides were varying in their chemical composition. Presence of the sugar shows that these were hetero/homo polysaccharides. IR analysis of the crude extracts for the presence of functional groups indicated a need for more purification of exopolysaccharides. Moreover the IR analysis along with histochemical studies showed the presence of hydroxyl, carboxyl and nitro groups. The physicochemical characteristics of the exopolysaccharides showed that the exopolysaccharides would be used in industrial applications. However, a thorough investigation is needed to determine their industrial application.

Key words: Bacteria, exopolysaccharides, high performance liquid chromatography, Infra-red spectroscopy, atomic absorption spectroscopy, flame photometer, histochemical studies

Introduction

Bacteria release exopolysaccharides in the environment as extracellula secretions in the form of capsules. Exopolysaccharides produced by bacteria are implicated in soil aggregation around roots, maintaining physiochemical characteristics of the soils. Characterization of polysaccharides in solutions is often difficult due to their chemical heterogeneity, multiplicity of linkages, high molar mass, broad molar mass distribution and high viscosity.

The role of exopolysaccharides in invasion is not yet clear. Exopolysaccharides may play a structural role, benefiting the bacterium by enabling attachment to surfaces, improving nutrient acquisition or providing protection from environmental stresses and host defenses. Alternately, exopolysaccharides may function as a signaling molecule, triggering a developmental response in the plant regulatory host defense responses (Gonzalez *et al.*, 1997). Other than their importance in soil, exopolysaccharides are also being used in the removal of heavy metals from industrial effluents and environmental media including potable water (Norberg and Persson, 1984).

Bacterial exopolysaccharides find applications in various fields like food, textile, paints, pharmaceutical, petroleum, health and agriculture as gelling, emulsifying, binding agents, protective collides etc. (Anonymous, 1975). Keeping in view their commercial importance present research work was undertaken to study the histochemical and physiochemical characteristics of these exopolysaccharides.

Materials and Methods

Isolation and extraction of exopolysaccharides: Isolation of exopolysaccharides produced by bacterial strains were made by pour plate and serial dilution methods using different sugar media (Heulin *et al.*, 1987). The exopolysaccharides were extracted according to the method of Hebber *et al.* (1992) and Ashraf *et al.* (1998).

Elemental analysis: Elemental analysis was carried out using standard method of Anonymous (1990).

Identification of functional groups: Infrared (IR) spectrophotometer was used for the identification of various functional groups in the exopolysaccharides samples.

Viscosity: Ostwald's viscometer was used to measure the viscosity of exopolysaccharides.

Analysis of carbohydrates: High pressure liquid chromatographic methods were used for the analysis of sugars in bacterial exopolysaccharides.

Histochemical study: Histochemical study was done to find certain structural groups in exopolysaccharides. For this purpose different dyes used were periodic acid as Pearse (1968), Toluidine blue (O' Brein and McCully, 1981) Alcian blue (Pearse, 1985) and aniline blue.

Results and Discussion

Physical state, solubility and pH: The results of physical parameters (state, solubility and pH) are shown in Table 1. EPS-10 and EPS-17 has amorphous form while EPS-133 is powdered. Physical appearance of a compound reflects its energy status. The crystalline form is energetically less stable than amorphous and more stable than powdered form. So it can be concluded that latent heat of fusion of the EPS-10 and EPS-17 was higher while it would be lower for EPS-133 which was in powered form. The physical state of the three exopolysaccharides would also be indicative of variability in chemical composition of the compounds (Sutherland, 2000). A similar solubility (in water) as well as pH 6 of the three exopolysaccharides was also an indication of the inherent chemical composition but it did not reflect that the compounds would be similar.

Viscosity: The values for viscosity of the three exopolysaccharides varied from 1.846 to 3.0 poise. A higher value for EPS10 showed that the chemical components of this exopolysaccharides were capable of creating a higher viscous solution than the other two. Among the three exopolysaccharides, EPS-133 showed the minimum (1.846) viscosity while the viscosity of EPS-17 (2.679) falls in between the two. The difference in viscosity is due to different internal structures of the compounds (Whistler and Be Miler, 1993).

Elemental analysis: Monovalent (Na, K) divalent (Ca, Mg) and polyvalent (Fe, Cu, Ni, Mn, Cd, Zn, Pb) elements were determined by flame photometer and atomic absorption spectrophotometer respectively. The results reveal that in polyvalent cations, Pb contents of the exopolysaccharides vary from 0.20 to 0.22 ppm, Mn from 0.04 to 0.22 ppm, Ni from 0.29 to 1.31ppm and Zn from 0.06 to 0.26 ppm (Table 2). The values for Cd were similar (0.05 ppm) for all the three samples. A comparison of the three exopolysaccharides showed that the Pb and Ni contents of the exopolysaccharides were similar but Mn and Zn were variable. There was a high affinity of the exopolysaccharides with Ni and least with Cd ion. Among the three exopolysaccharides, EPS-17 was more reactive with the heavy metals. Similarly, the data of divalent contents of three

Table 1: Physical state of exopolysaccharides

Sample	State	Solubility	pH	Viscosity (in poise)
133	Powdered	Water	6	1.846
10	Amorphous	Water	6	3.000
17	Amorphous	Water	6	2.678

Table 2: Elemental analysis (ppm) of exopolysaccharides

Samples	Pb	Mn	Cd	Zn	Ni	Ca	Mg	Fe	Na	K
133	0.20	0.04	0.05	0.06	0.29	6.25	58.0	2.41	220.0	60.0
10	0.21	0.06	0.05	0.07	1.16	5.75	50.0	3.78	100.0	15.0
17	0.22	0.22	0.05	0.26	1.31	13.5	138.0	9.89	300.0	4.0

Table 3: High performance liquid chromatographic analysis

	Component	Amount (mg ml^{-1})
EPS-133	Fructose	0.1559
	Polysaccharide	3.7898
EPS-10	Fructose	0.2417
	Glucose	0.0029
	Maltose	0.0214
	Polysaccharide	9.2933
EPS-17	Fructose	12.1054
	Glucose	0.3753
	Polysaccharide	7.7554

Table 4: Functional groups detected by IR spectrophotometer

	Wave number (cm^{-1})	Functional group
EPS-10	1630	-C=0
	3200-3400	-OH
	985-995	-CH
	1625	-C=C
EPS-17	1320-1340	-NH
	1625	-C=C
EPS-133	1600	NO_2
	1000	-S=O
	1400	$-SO_2$

exopolysaccharides presented the Ca content from 5.75 to 13.5 ppm, Mg from 5.0 to 138 ppm and Fe from 2.41 to 9.89 ppm. Among the divalent cations the higher and maximum values were observed for Mg content. A comparison of the three exopolysaccharides presented a higher affinity of the EPS-17 with divalent cations and Fe content. All the three exopolysaccharides were processed through similar procedure, a variation in interaction of the exopolysaccharides with polyvalent and divalent cations as well as with iron could be attributed to inherent ability and structure of the exopolysaccharides to interact with the metallic ions (Pirog, 1997). By comparing the inorganic composition of three exopolysaccharides with respect to K and Na concentration of Na ions are found to be higher than K. EPS-133 showed higher conc. of K (60 ppm) while EPS-17 have maximum concentration of Na (300 ppm).

Inorganic composition of the microbial exopolysaccharides varies with the chemical composition of surrounding medium and affects the physical and biological functioning and structure of the microbial biofilms (Costerton, 1999). A higher affinity of the EPS-17 with the metallic ions indicated the ability of this biopolymer to extract metals from the surrounding medium. The presence of inorganic cations also shows the ability of the exopolysaccharides to attach with the substrates. Divalent and polyvalent metallic ions like Ca, Mg and Fe affect the intermolecular bonding and structural integrity of the biofilms as well as cause the precipitation of exopolysaccharides. Fletcher and Floodgate (1973) suggested that divalent cations, especially Ca can form bridges between negatively charged substrates and the presence of Lanthnum decrease the attachment due to its inhibitory effect for Ca bonding with the biofilm exopolysaccharides.

Carbohydrate analysis: High performance liquid chromatographic studies of exopolysaccharides indicated a wide variation of sugars and polysaccharide contents among the three microbial exopolysaccharides. Besides the higher concentration of polysaccharide the analysis of EPS-133 showed the presence of fructose and higher amount of exopolysaccharides. For EPS-10 maltose, glucose and fructose were detected with a higher content of exopolysaccharides. In contrast to EPS-133 and EPS-10 microbial exopolysaccharides, the HPLC analysis for EPS-17 showed three peaks indicating the presence of glucose and fructose and a high content of exopolysaccharides. A comparison of three microbial exopolysaccharides presented a higher exopolysaccharides content (9.2933mg ml^{-1}) for EPS-10 followed by EPS-17 (7.7554mg ml^{-1}) and EPS-133 (3.7898mg ml^{-1}) (Table 3).

The results obtained are in harmony with the findings of Grobben *et al.* (1997) who also found that the high molecular weight fraction of the purified exopolysaccharides have a sugar composition of galactose, glucose and rhamnose in the molar ratio of 5:1:1, whereas the low molecular weight fraction contained galactose, glucose and rhamnose in the molar ratio of 11:1:0.4.

Infra-red studies: The results pertaining to IR studies are shown in Table 4. The IR analysis of the crude extracts of the three exopolysaccharides showed a wide variation of structural groups (Micheli *et al.*, 1999). EPS-10 showed stretching at 1630, 3200-3400, 985-995 and at 1625 cm^{-1} indicating the presence of carbonyl (-C = 0), hydroxyl (-OH), alkane (-CH) and aromatic conjugated (-C = C) groups. In contrast to EPS-10, EPS-17 showed fewer and variable stretches. A stretch at 1320-3140 cm^{-1} represented the presence of amide hydrogen bonded (-NH) group, while a stretch at 1625 cm^{-1} indicated the presence of conjugated (-C=C) groups. Various IR stretches of the EPS-133 showed the presence of NO_2 aromatic (stretch at 1600 cm^{-1}) and diester linkage groups of - S=0 and -SO_2 (stretch at 1000 cm^{-1} and 1400 cm^{-1}) respectively. A comparison of functional groups presents that the EPS-10 having a higher number of variable functional groups was more complex than the other two exopolysaccharides. The difference in the presence of a larger number of functional groups also makes it clear that the crude extracts of bacterial exopolysaccharides have a large number of other chemical compounds along with polysaccharide and sugars as the major constituents.

Histochemical studies: Histochemical analysis presented a strong indication of the presence of anionic sites, carboxyl groups (Rogers, 1979) and proteins (Corpe *et al.*, 1976). All the three exopolysaccharides showed that the extent of anionic sites, carbonyl groups and protein content was higher and maximum in EPS-17. The presence of 1, 2 diol groups was indicated with periodic acid stiffs reagent reaction and it was found more in EPS-10 than other two exopolysaccharides.

Exopolysaccharides (EPS) are the products of the microbial metabolism. Physicochemical analysis indicated that the exopolysaccharides were varying in their chemical composition. Presence of the sugars shows that these were hetero or homopolysaccharides. IR analysis of the crude extracts for the presence of functional groups indicated a need for more purification of the exopolysaccharides. Moreover, the IR analysis along with histochemical studies shows the presence of hydroxyl, carboxyl and nitro groups indicated that the exopolysaccharides would be used in industrial applications. However, a thorough investigation is needed to determine their industrial application.

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