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A HYDROPHILIC AND HALOPHILIC BIOPOLYMER PRODUCED BY SOIL BACTERIUM CMG1447mp

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ABSTRACT

The extracellular polymeric substance produced by dry soil isolate *Pseudomonas putida* CMG1447mp was characterized for hydrophilic and halophilic activities. CMG1447mp was found to produce a similar polymer from either fructose or glucose or sucrose and polymer from each carbon source was an anionic heteropolysaccharide containing glucuronic acid, D-glucose with higher concentration, D-mannose and L-rhamnose. The polymer produced from different carbon sources was found to express high hydrophilic and halophilic activities in both hot and cold environments. Bacterial origin polymer was hygroscopic, heat stable and can function as water reservoir.

Key words: Heteropolysaccharide, hygroscopic, halophilic, hydrophilic.

INTRODUCTION

In the age of modern science and technology, various biotechnological methods are needed at priority basis to overcome the challenging global problems of poverty, scarcity of agriculture lands, deficiency of water, dryness, aridity, salinity and pollution. Another ecological global concern is fertile soil erosion and expanding desertification at the rate of about 60,000 Km^2/year (Kurane and Nohata, 1994). More than 90% of world's water is seawater and more than half of the world's groundwater supplies are saline, and this proportion is increasing as demand for water outstrips supply and salinity limits crop production, further the rate of rain fall approximately in 100 countries of the world, with a population of one billion people is on average, less than 150 to 200 mm, which restricts agriculture (Moazami, 2001). According to the estimates of United Nations Environment Programme, revival of these lands and their transformation to fertile and cultivable grounds requires 10 to 20 billion US Dollars per annum, and demands 20 years of expenditure (Moazami, 2001). Now synthetic hydrophilic articles being used for greening and sanitary purposes are not degradable and deleterious to environment, health and soil fertility (Kurane and Nohata, 1995, Sutherland, 2001). However natural hydrophilic polymers are susceptible to degradation and the adverse effects would be negligible. Hence, the development of biodegradable alternatives are strongly desired (Kurane and Nohata, 1995). Against synthetic hydrophilic polymer, natural hydrophilic compounds such as bacterial water absorbing exopolysaccharides are both chemically and biologically degradable into nontoxic, energy storing, useful disaccharides and monosaccharides, hence do not pose a secondary pollution environmental hazards (Bartello *et al*., 1966; Nguyen and Schiller, 1989; Warren, 1996). Moreover bacteria-derived polysaccharides have halophilic activity which reduces the salinity of soil. Along with accompanying cells, they act as water absorbing biological fertilizer, which not only fertilizes the soil but also stabilize the soil and provides the required water to the plant (Moazami, 2001). The concentration of bacteria and production of water-absorbing biopolymer by bacteria play a fundamental role in absorption and retention in the soil and around the plant roots (Moazami, 2001). A huge amount of water is currently needed for the irrigation of greening the deserts, most of the irrigated water is immediately disappeared into depth, and into air at high temperature, thus causing injury from salts (Moazami, 2001). By employing these biological methods, arid, dry, saline and desert areas could be transformed into fertile agriculture lands and soil erosion would be prevented which in turn may maintain the fertility of soil. In addition, man planted forest can also be beneficial. Once a green bed is substituted in nature, it will cause fundamental modifications to environment and ecosystem over the years, the environment will remain green and fertile. The aim of present study was to estimate the hydrophilic and halophilic characteristics of exopolysaccharide produced by *Pseudomonas putida* CMG1447mp isolated from soil.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Gram negative soil bacterium CMG1447mp was identified by using DESTO QTS-24. Minimal medium used for the production of biopolymer was described previously (Muhammadi *et al*., 2006). Bacterial strain CMG1447mp

was grown over night in above medium at 30°C. 1ml of seed culture was inoculated into 2000ml flask containing 1litre above medium, and incubated at 30°C for 5 days.

Extraction of biopolymer

Extraction and purification of biopolymer from 5 days old culture was carried out according to the method as described previously (Muhammadi et al., 2006). The polymer produced using either fructose or glucose or sucrose as carbon source was recorded as bp**F**, bp**G** and bp**S** respectively.

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 the method of Bradford (1978) with bovine serum albumin standard, 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 of biopolymer

1mg of sample was dissolved in 500µl 2M Trifluoro-acetic acid (425µl double distilled water + 75µl TFA) into an airtight vial and hydrolyzed at 120°C for 3hrs. Then the hydrolyzed sample was dried over night in a Wheaton dry-seal vacuum over $CaCl_2$. Dried hydrolysate was resuspended in 50µl of distilled water for paper chromatography.

Paper chromatography

Descending paper chromatography was carried out on Whattman paper no. 3 for 18 hrs at room temperature with solvent system consisting of BuOH: Acetic acid: water (4:1:5), shaken well and top layer was used. Chromatographic papers were dried and stained with alkaline silver nitrate reagent (George and William, 1974).

Measurement of moisture and ash content

Moisture, ash (inorganic) and organic contents of each sample (bp**F**, bp**G** and bp**S**) were determined by the methods of Horwitz (2000).

Measurement of hydrophilic activity

Hydrophilic activity of biopolymer was determined in terms of water absorption and water retention capacities according to "tea bag method" (Kurane and Nohata, 1994). Absorption and retention capacities were optimized under different length of stipulated time (1, 2, 4, 12, 24, 48, 72hrs and 1, 2, 5, 10, 15, 20days respectively) at different temperatures (10, 20, 30, 40, 50 and 60°C).

Measurement of halophilic activity

Accumulation of water soluble NaCl (0.89, 2, 4, 6, 8, 10 and 15%) by bacterial polymer (100mg) was carried out according to method of Kurane and Nohata, (1994). Further, percolated sample was heated (80-110°C) in OSK 9500B electric oven until a constant weight was reached for complete evaporation of H_2O. Then halophilic activity was calculated in term of NaCl accumulation (%) as follow:

$$\text{NaCl accumulation (\%)} = \frac{W_a - W_b}{W_c} \times 100$$

W_a: Dried weight of sample after absorption; **W_b**: Weight of sample before absorption; **W_c**: Weight of NaCl supplied.

RESULTS

Quantification of biopolymer

After purification, an average of 3.5-4g lyophilized, homogeneously white polymer was obtained from one liter minimal broth, with either fructose or glucose or sucrose (data not shown). Lyophilized polymer (bp**F**, bp**G** and bp**S**) was found to contain an average content of 92.65% neutral sugars and 7.25% uronic acid while colorimetric reactions for protein, phosphorus and lipid were negative.

Hydrolysis of biopolymer

As result of acid hydrolysis of bp**F**, bp**G** and bp**S**, four spots were developed on paper chromatogram whose R_f values were in agreement with glucuronic acid, D-glucose, D-mannose and L-rhamnose. D-glucose aligning spot released was more concentrated as compared to rest of three other spot (Table 1).

Table 1. Monomer composition of biopolymer released by acidic hydrolysis

Standard monosaccharides	R_f values	Tentative assignment of spots separated		
		bpS	bpF	bpG
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			
L-Rhamnose	44.47	+	+	+

+: Light spot., ++: Dark spot., **bpF**, **bpG** and **bpS**: biopolymer produced from fructose, glucose and sucrose respectively.

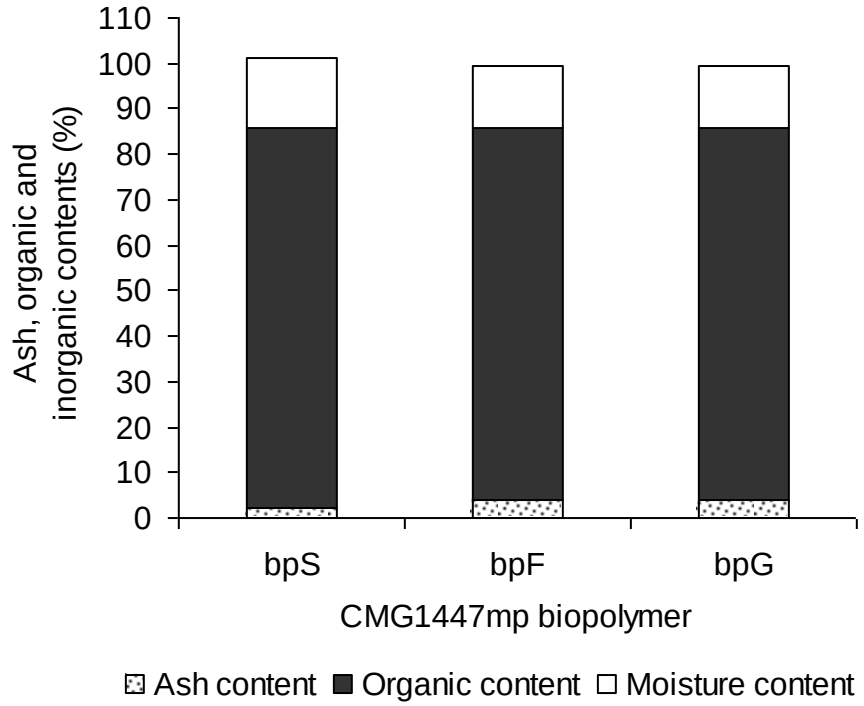

Fig. 1. Distribution pattern of ash, organic and moisture contents in bp**F**, bp**G** and bp**S** produced by *Pseudomonas putida* CMG1447mp.

Table 2. Optimum capacities of biopolymer for water absorption and retention

Activity(%)	length of	Temperatures (oC)					
	time	10	20	30	40	50	60
Water	1hr	115	205	256	309	343	370
Absorption	2hrs	185	327	352	385	430.5*	430*
	4hrs	203	327	425	430*	423	429
	12hrs	233	375	430*	430	428	430
	24hrs	241	425*	430	428	429.3	427
	48hrs	265*	423	427	428	429	430
	72hrs	263	425	430	429	427.78	430
Water	1day	98	97	95	83	77	52
Retention	2days	96	94	90	78	70	47
	5days	91	88	85	67	60	40
	10days	87	81	78	53	44	36
	15days	82	80	78	46	38	36
	20days	82	81	78	41	38	36

*: Optimum water absorption at certain temperature.

Table 3. Optimum accumulation of NaCl in biopolymer

Supplied amount	Amount of NaCl (%) accumulated/g of biopolymer		
of NaCl (%)	bpS	bpF	bpG
0	0	0	0
0.89	20	19.5	19.5
2	23	22.7	22.5
4	30	30	29.8
6	35	34.6	35
8	38	38	38
10	40	40	40
15	40	40	40

Measurement of moisture and ash content

The vacuum-dried samples bp**F**, bp**G** and bp**S** contained average content of 81.88, 81.85 and 83.84% organic content respectively. Moisture content was, 13.72, 13.86 and 15.16% respectively. While ash content was 4.03, 3.75 and 2.05% (Fig. **1**).

Measurement of hydrophilic activity

No considerable differences were observed in water absorption and retention capacities among bpF, bpG and bpS, therefore average capacities were calculated (Table 2). It was observed that at 10, 20, 30, 40, 50 and 60^{o}C temperature, bacterial polymer attained its maximum water absorption in 48, 24, 12, 4 and 2hrs respectively (Table 2). Similarly at 50 and 60^{o}C the activity was attained in 2hrs. At 10 and 20^{o}C, more than 80% of supplied water was retained. More than 70% was retained at 30^{o}C. Similarly at 50 and 60^{o}C, more than 35% water was retained. Although a slow rate of evaporation was observed up to 15 days but after that till 20th day no further loss of supplied water was observed (Table 2).

Measurement of halophilic activity

The present bacterial polymer had accumulated the water soluble NaCl molecules even from a solution of 0.89% concentration. It could accumulate up to 40% of supplied NaCl. As concentration of NaCl was increased up to 10%, its NaCl accumulation capacity had also increased but in solution where NaCl concentration was higher than 10% no further NaCl accumulation was observed (Table 3).

DISCUSSION

Soil bacterial strain CMG1447mp identified as *Pseudomonas putida* was found to produce a hydrophilic, halophilic degradable anionic exopolysaccharide in minimal broth. After lyophilization physically a homogenous and pure white polymer was obtained. Quantitative analysis showed that the bacterial polymer produced either from fructose or glucose or sucrose as sole carbon source was anionic polysaccharide in nature. Acid hydrolysis has revealed that the present polymer was an anionic heteropolysaccharide made up of glucuronic acid, D-glucose with higher concentration, D-mannose and L-rhamnose (Sutherland, 1997). It was also showed that CMG1447 could synthesize a similar polymer from three different carbon sources. When the purified polymer (0.5%) was used as sole carbon source in the minimal medium, bacterial strains from CMG stock such as *Pseudomonas putida, Pseudomonas stutzeri, E.coli* and five unidentified gram negative bacterial strains showed a visible growth which suggested polymer biodegradation (data not shown). Since, polysaccharides are insoluble substrates and microorganisms utilize them by producing extracellular glycosidic hydrolases, free or cell surface associated, that convert the polysaccharides to soluble products that are transportable to the cells (Bartello *et al*., 1966; Dunne *et al*., 1985; Nguyen and Schiller, 1989; Warren, 1996). After the physical composition analysis high amount of moisture and organic content contained in vacuum and freeze dried polymer has shown that the current bacterial polymer is a hygroscopic organic compound. The polymer was found to absorb water at both lower and higher temperatures, and as the environmental temperature was raised, maximum water absorption was achieved earlier. Perhaps, this was because that increasing temperature enhances the Brownian movement of water molecules due to which the rate of penetrating activity of water molecules into biopolymer was increased. In the presence of lab scale desert hot environmental conditions, the current biopolymer could keep a considerable amount of supplied water, this suggested that its stable affinity towards water. After complete evaporation of water absorbed by polymer in the presence of NaCl, an increased dried weight of polymer has been observed. This has suggested that deposition of NaCl might have been carried out during absorption of water. Therefore it is believed that the polymer could demonstrate dual activities i.e. water storage and salt accumulation, so it could be useful for revival of saline areas (Moazami, 2001). After maximum accumulation of NaCl no further deposition of salt was observed. The biopolymer is extracellular and released in the culture medium, therefore presence of salt does not cause any damage to the polymer. According to Moazami (2001) water and salts are absorbed in thin channels or inter molecular spaces of bacterial biopolymer superwater absorbent. Further, polysaccharides are high molecular weight substances that have a large binding capacity for other substances (Fredrik, 1997). The present bacterial origin polymer was high molecular weight acidic polysaccharide, so when it was exposed to water, it might establish a hydrophilic interaction with water and a halophilic interaction with water soluble ionized sodium chloride salt (NaCl) (Fredrik, 1997). Another possible logical reason is that CMG1447mp was isolated from dry land soil, therefore, highly hydrated (hydrophilic) exopolysaccharide layer or network surrounding the cell had to be produced to protect the organisms from adverse environmental stresses and lethal desiccation, (Sutherland, 2001; Whitfield, 1988). Hence it is concluded that the present extracellular polysaccharide shall be equally useful for agriculture lands with

diversified environmental temperatures, salinity and will not contribute to pollution, thus, will be much safer for soil, water, plants, human being and even microbial flora (Sutherland, 2001).

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