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Production of Biopolymers by *Pseudomonas aeruginosa* Isolated from Marine Source

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ABSTRACT

Two bacterial strains, Pseudomonas aeruginosa *CMG607w and CMG1421 produce commercially important biopolymers. CMG607w isolated from the sediments of Lyari outfall to Arabian Sea synthesize the mcl-polyhydroxyalkanoates from various carbon sources. The production of PHAs was directly proportional to the incubation periods. Other strain CMG1421, a dry soil isolate, produced high viscous water absorbing extracellular acidic polysaccharide when it was grown aerobically in the minimal medium containing glucose or fructose or sucrose as sole source of carbon. The biopolymer had the ability to absorb water 400 times more than its dry weight. This property was superior to that of currently used non-degradable synthetic water absorbents. It acted as salt filter and had rheological and stabilizing activity as well.*

Key words: Biopolymers, exopolysaccharides, hydroabsorbent, mcl-polyhydroxyalkanoates, Pseudomonas aeruginosa, Sodium gluconate

INTRODUCTION

Biopolymers such as the Polyhydroxyalkanoates (PHAs) and the Exopolysaccharides (EPS) are diverse and versatile class of the materials that have potential applications in virtually all sectors of the economy. In response to the increasing public concern about the harmful effect of the petrochemical derived plastic materials in the environment, many countries are looking for alternate source such as biodegradable plastic materials. These materials must retain the desired material properties of the conventional synthetic plastics, and should be able to degrade on disposal. PHAs are polyesters of various hydroxyalkanoates, which are synthesized by the microorganisms. They are considered to be strong candidate for biodegradable polymer as they possess all the properties of synthetic plastic and when discarded are completely degraded to water and carbon dioxide by microorganisms (Lee, 1996). The fluorescent *Pseudomonas* belonging to the rRNA homology group I have been reported to synthesize and accumulate large amount of medium chain length polyhydroxyalkanoates (mcl-PHA) consisting of various saturated 3-hydroxy fatty acids with carbon ranging from 6 to 14 carbon atoms and they act as energy storage compound. The PHA comprising of medium chain monomers (6 or more carbon length) is more elstomeric and may contain unsaturated carbon bonds. They are more conducive for coating and film materials and offer greater possibilities for the chemical modifications (Anderson and Dwes, 1990).

The modernization in the life style has increased the use of various kinds of the sanitary products including disposable baby diapers. Most of the

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water absorbing materials being currently used in these products are made up of synthetic organic compounds and their derivatives such as polyacrylate, polyacrylamide, acrylic acid and acrylamide, etc. These synthetic absorbing polymers are nondegradable, harmful (Merck Index) and carcinogenic (Ryuichiro and Nohata, 1994) for human beings. Hence, when these are discharged and disposed, they remain in the environment for long periods, thus contributing to the environmental pollution (Ryuichiro and Nohata, 1994). However, the natural water absorbing compounds such as the bacterial exopolysaccharides are environment friendly, non-toxic to human, animals and microorganisms and are degradable by the microorganisms. Present study was conducted to isolate the bacterial strains able to synthesize commercially important biopolymers, to optimize their production and to study the chemical nature of these polymers.

MATERIALS AND METHODS

Isolation, purification and cultural conditions

A sample of the sediment from the western beach near the Lyari outfall was collected from Karachi coast (around 47.21°N 67.10 °E). The bacterial strain was isolated, purified and coded as CMG607w, while the strain CMG1421 was isolated from the dry soil, Karachi University; it was also purified and both were preserved in 20% glycerol at –70°C. The strains were identified by using the API kit and were found to be *P. aeruginosa.* Strain CMG607w was maintained at 30°C in artificial sea water (ASW) (Kuniho and Kurane, 1999) in 1 L of distilled water, supplemented with 0.5 g tryptone and 10 g carbon source (glucose/sucrose/ sodium acetate or sodium gluconate). For the PHA synthesis, same cultural conditions were used and the incubation time was increased from 24 to 120 h at 30°C and 200 rpm. For the synthesis of the hydroabsorbent polysaccharide, CMG1421 was grown in the minimal medium as described by Ryuichiro and Nohata (1994), supplemented with 20 g/L carbon source. After growing in the same minimal medium, 1 ml (48 h old) seed culture was inoculated into 1 L minimal medium and incubated at 30°C for 15 days.

Extraction and purification of Biopolymers

The lyophilized cell material was used for the PHA extraction with chloroform at 65° C for 4 h in the screw cap bottles. The extraction was done three times from the same material. The cell debris were removed by passing through a cellulose filter and the chloroform solution was concentrated by using a rotary evaporator (BUCHI Rotavapor R-114, Switzerland). The polymer was precipitated by pouring the chloroform solution (1 volume) into ethanol (5 volumes). The precipitated polymer was separated from the liquid by the filtration and was dried by exposure to hot air. The precipitation was repeated for further purification and used for the chemical analysis.

The viscous culture broth of CMG1421 was diluted (Kuniho and Kurane, 1999) with half volume of sterilized distilled water and placed in shaking incubator (30°C) for one hour. The bacterial cells were sedimented by centrifugation at 10,000 rpm for 30 min at 4°C using KOKUSAN H-200nR refrigerate centrifuge. Trichloro acetic acid (TCA) 5-10% was added to the cell-free supernatant to precipitate the extracellular protein fractions. Then it was centrifuged at 9,000 rpm for 30 min at 4°C to collect the protein fractions. The clear supernatant was added to equal volume of absolute ethanol (Hiroyasu et al, 1992). The precipitates were collected around the glass rod. The dissolution and precipitation process was carried out four to five times until the uniform white precipitates were obtained. The sample was dried in the wheaten dry-seal vacuum desiccators. The crude hydroabsorbent was dialyzed against the distilled water and lyophilized.

Quantification and structure elucidation of PHA

The Quantification of the PHA produced by the CMG607w was determined by Slepecky and law method (1961). For the structure elucidation, the purified PHA was dissolved in the chloroform and layered on the KRS-5 window. After the evaporation of the chloroform the PHA polymer film was subjected to the FTIR (Fourier transmison infra red) analysis. The infrared spectra was recorded on JASCO 320-A spectrometer with a beam condenser and the spectra range used was 5000 -330 cm^{-1}. The purified PHA was also analyzed by using 300-MHz ^{1}H-NMR (proton-nuclear magnetic resonance) spectrophotometer. A 0.5 % (w/v) polymer in (*d*-chloroform) $CDCl_3$) spectrum was recorded at temperature 298 K. ^{1}H-

NMR peaks areas were determined by spectrometer integrations.

Chemical composition of EPS

The chemical composition of the dialyzed samples from the CMG1421 was determined in terms of total contents of the following; carbohydrate by anthrone-sulphuric acid assay (Spiro, 1966) and phenol-sulphuric acid assay(Kochert, 1978), amino sugars by Elson-Morgan assay (Dey and Harborne, 1990), protein by Bradford method (1978) , phosphate by George et al method (1974), and acidic sugars (uronic acid) by Carbazol assay (Bradford, 1978).

Water absorption capacity

The water absorption capacity of the purified bioabsorbent was determine by "the tea bag method" (Ryuichiro and Nohata, 1994). The dried and labeled tea bag was filled with 20 mg sample, and each of them was immersed into 200 ml distilled water and NaCl solutions contained in beakers and left for 2, 4 and 6 h. Besides the biopolymer, ten other controls samples i.e. xanthan gum, sodium alginate, agar, cellulose, ion exchange resin, anionic high-polymer absorbent (Diafloc IncP-MP), silica gel, high grade synthetic high-polymer absorbent (Sumika Co., Gel S-50), soil and empty tea bags were also tested for their water absorption capacity. The maximum water absorption capacity (g) per gram of dried samples was calculated in terms of average.

Moisture absorption capacity

The moisture absorption capacity was also determined by the method of Ryuichiro and Nohata (Ryuichiro and Nohata, 1994). Wheaton dry-seal vacuum desiccators containing the saturated solutions of magnesium chloride was used at relative humidities of 61.8% while being stored at 37^{o}C in an incubator for 2, 6, 24 and 72 h.

Water retention capacity

The Water retention capacity was measured by the "glass column method" (Ryuichiro and Nohata, 1994). The dried (100 mg) hydroabsorbent synthesized in the presence of sucrose, sodium alginate, xanthan, synthetic acrylamide high polymer was mixed with 100g of dried sand and packed in each column. The amount (g) of water retained by each sample was estimated for 10 min, 1, 10 and 24h at 60^{o}C with 50-60% relative humidity.

Moisture retention capacities

A100 mg of the vacuum dried and heat dried (110^{o}C) hydroabsorbent (in the presence of sucrose) sample, $CaCl_2$, silica gel, glycerin, urea, anionic polymer, were weighed. 30 µl distilled water was added to each sample and then left at 60^{o}C. Each sample was weighed after 1, 2, 5, 10 and 15 days and moisture retention capacities of each sample were calculated as average of three adjacent values (Ryuichiro and Nohata, 1994).

RESULTS

PHA accumulation and chemical analysis

P. aeruginosa CMG607w was able to utilize glucose, sucrose, sodium acetate and sodium gluconate as carbon source under the aerobic conditions. Strain CMG607w grew efficiently in the chemically defined media supplemented with sodium gluconate and showed exponential phase of about 25 h with high cell density while in the presence of glucose/sodium acetate/sucrose, the exponential phase was of 20 h with low cell density. The PHA extracted from CMG607w was in the form of white precipitates in the presence of all the tested carbon sources but in the presence of sodium gluconate, the amount of PHA produced was higher as compared to that of other carbon sources.

Table 1 - PHA synthesis by CMG607w from sodium gluconate in shake flask cultures.

Time	pH	Lyophilized cell wt (mg/L)	PHA con. (mg/L)	PHA content (wt%)
24	6.2	243	56	23.0
48	6.0	690	213	30.8
72	5.3	889	376	42.3

In the presence of 1% sodium gluconate, it synthesized 56 mg/L of PHA after 24 h while the maximum production of PHA was 376 mg/L after the 72 h. With the increase in the incubation time, the PHA content in the cells tended to increase and reached to 42.3 wt% of the dry cell mass (Table 1). The purified PHA showed strongest band at 1735 cm^{-1} for the carbonyl easter group in the FT-IR spectra (Fig.1).

The methylene (CH_2) vibration near 2925cm^{-1} was also observed. The purified PHA isolated from the cells grown in the media with sodium gluconate when analyzed by using 300-MHz ^{1}H-NMR spectrometer (Fig. 2) showed that the mcl-PHA was the major composition. The integration values agreed well with the proposed polymer structure (Ohyoung et al, 1995). The ^{1}H-NMR spectra showed the characteristic methyl, methylene and methine protons of the PHA. The most upfield triplet of the methyl appeared at δ 0.87(J=6.6Hz) and the octet appearing at δ 2.42-2.62(J_{AX} =5.7Hz, J_{BX} =7.4Hz, J_{AB} 15.5Hz) was due to methylene (H-a). A broad singlet of 18 ^{1}H integration appearing at δ 1.25 was assigned to (H-d). In the same spectrum, one broad singlet of two proton integration resonated at δ 1.58 and was assigned to H-e. The down field multiplet resonating at δ 5.23 was due to the only methane. All the chemical shifts were confirmed by comparison with the reported data (Ohyoung et al, 1995).

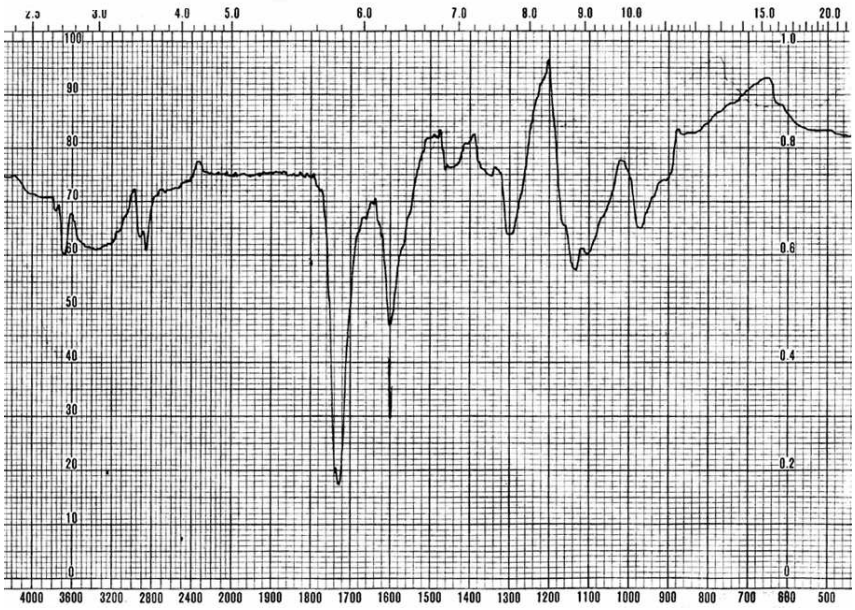

Figure 1 - FT-IR Spectra of purified mcl-PHA from CMG607w

EPS production and chemical analysis

The ethanolic precipitation showed that the insoluble precipitates were flocculent of high molecular weight compounds. In the absence of urea, the viscosity of the culture was significantly decreased which has suggested that urea enhanced the synthesis of the polymer. The analysis of the chemical composition of the biopolymer produced using glucose/ fructose or sucrose showed that the total carbohydrates were 95.73, 96.86 and 97.06 %; uronic acid was 2.15, 2.91 and 2.78 % respectively. A trace amount of PO_4 was also detected in all the samples (Table 2).

Table 2 - Chemical composition of hydroabsorbent biopolymer (contents expessed as µg%w/w).

Samples	Carbohydrate	Protein	Amino Sugars	Uronic acid	PO_4
bpS	95.732	-	-	2.15	0.728
bpF	96.856	-	-	2.908	0.515
bpG	97.057	-	-	2.776	0.065

bpS: Biopolymer obtained when sucrose was used as carbon source
bpF: Biopolymer obtained when fructose was used as carbon source
bpG : Biopolymer obtained when glucose was used as carbon source

Table 3 - Water absorption (%) of various absorbents per gram of sample.

Without NaCl			**NaCl (%)**			
Groups	Sample	Dst.water	0.89	2	4	6
Test group	bpS	432.65	245.31	185.33	139.13	85.0
	bpG	426.5	200.11	153	123	74.54
	bpF	422.0	205.56	145.74	130.44	70.78
Control group	High grade synthetic	244.72	112.51	92.81	67.0	39.75
	Anionic synthetic	348.33	135.54	103.53	85.33	47.87
	Alginate	78.83	47.45	34.35	28.78	27.68
	Xanthan	77.0	45.56	34	30.77	27.89
	cellulose	45.75	35.23	24.95	23	20.42
	Ion exchange resin	9.1	5.68	2.34	2.0	2.2
	Soil	2.44	2.33	2.43	2.12	2.0

Table 4 - Retention capacity (%) of supplied water to mixture of hydroabsorbent and Sand under different time intervals.

Samples	10min	1h	12h	24h
bpS	97	87	51	38
Xanthan	10	5	0	0
Cellulose	11	4	0	0
Alginate	12	8	8	8
Polyacrylamide	17	3	3	0
Synthetic high polymer absorbent	9	5	5	1

Hydroabsorbent properties of EPS

Results of the water absorbing properties of the biopolymer revealed that the biopolymer absorbed 400 times more water than its own weight which was much higher when compared with that of commercially available water absorbing compounds, i.e. high grade synthetic high-polymer absorbent and anionic high-polymer absorbent whose absorption capacities were 244 and 348 times of their dried weight, respectively. The water absorption capacity of the present hydroabsorbent was 3-5 times higher than that of commercially available polysaccharide gums, such as xanthan, alginate and cellulose (Table 3). When the saline

conditions were developed, the water absorption capacity was decreased with an increase in the concentration of NaCl (Table 3) but this reduced capacity was still superior to the absorption capacity of the control samples.

In the presence of 61.8% moisture, the bacterial hydroabsorbents (bpS, bpF, bpG) were capable to absorb 26-31% moisture, which was also superior to those of the control polysaccharides (xanthan, alginate and cellulose). $CaCl_2$, and Glycerin showed higher moisture absorption than the test hydroabsorbent but $CaCl_2$ became liquefied after the saturation at 72 h this was, however, not observed with under study bioabsorbent (Fig. 3).

When the hydroabsorbent polymer and synthetic water absorbents with the soil were subjected to the lab scale desert environmental conditions (50-60% relative humidity, 60^{o}C) for the retention capacity of supplied water, it was observed that the hydroabsorbent polysaccharide could retain 38% of supplied water for 24 h (Table 5). While the synthetic high absorbent and polyacrylamide could not retain the supplied water in same conditions, only alginate could retain 8% water (Table 4).

DISCUSSION

The use of the synthetic polymers has become a common practice in our daily life. These polymers have made the life easy, e.g. the use of plastic bags, bottles, tissues papers, baby diapers, etc. On one hand, they have made the life easy but on the other hand, they are responsible for a great contribution in the environmental pollution. On disposal, these polymers do not degrade and remain in the environment. Realizing this situation, the scientists are now looking for the alternate source such as biopolymers.

The production of PHA by CMG607w showed that the PHA was accumulated in large quantities in the cells (unpublished data), after the cessation of growth and nutrient unbalance conditions (Lee, 1996). PHA accumulation after 24h was 23 wt% of cell dry mass while after 72 h of incubation it reached to 42.3 wt% of cell dry mass. Purified PHA showed strongest band at 1735 cm^{-1}, in comparison, to PHA being produced *A. zotobacter vinelandii* and *Pseudomonad mendocina* their carbonyl bands (C=O) at 1732 cm^{-1} (Hong et al, 1999). In the presence of sodium gluconate mcl-PHA was identified by H^{+}NMR, hence it was concluded that CMG607w accumulated a polymer with medium chain length as the major constituent when the cells were grown on sodium gluconate (Huijbert et al, 1992). The strains producing 40% or more mcl-PHAs are considered to be the good candidates for the commercial exploitations (Harbak, 1992). The CMG607w could have the economical advantage that it used the inexpensive carbohydrate derived substrate (gluconate) as the sole carbon source for the mcl-PHA production.

The CMG1421 produced an extracellular polysaccharide in the minimal broth. The polysaccharide made the broth very viscous, which consisted of mainly carbohydrate. Further more, the chemical composition was suggested that bacterial biopolymer was acidic polysaccharide in nature (Table 2). The dried weight of extracellular biopolymer was found in the range of 2-4g per liter of the broths. The polymer was found to be insoluble in tap water, hot water (60^{o}C), distilled water and saline; this suggested that the bacterial biopolymer was tolerant against different environmental conditions (Table 5).

This polysaccharide absorbed 3-4 hundred times more water as compared to its own weight which was superior to that of compounds as bioabsorbents used as controls (Table 3). It showed that the polymer was insoluble hydrophilic compound and acted as water reservoir. In the presence of NaCl, reduction was observed in the water absorbing capacity but the capacity of hydroabsorbent was still significant as compared to those of controls. The reason for the reduction in the water absorption of the polymer in salty conditions could be that this polymer acted as a salt filter and sections of the polymer eventually were saturated with the salt, hence, a reduction in the water absorption was observed (Moazami, 2001).

Table 5 - Moisture retention capacities (%) of different absorbents under relative temperature 60°C.

Sample	Moisture retention at given intervals (days)					
	0	1	2	5	10	15
bpS	100	76	70	72	58	47
$CaCl_2$	100	78	73	68	60	45
Silica beads	100	50	40	25	17	12
Glycerin	100	62	44	29	13	13
Urea	100	33	7	7	2	2
Anionic polymer	100	41	25	22	22	22
Plastic cup	100	0	0	0	0	0

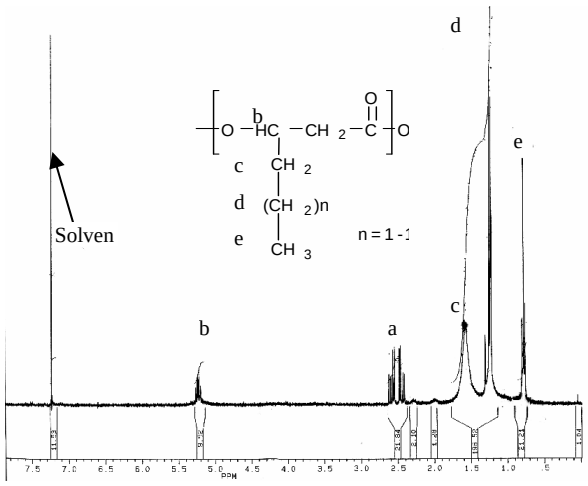

Figure 2 - The 300-MHz ^{1}H-NMR spectrum was recorded in *d*-chloroform of the PHA produce by *P. aeruginosa* CMG607w using Na-gluconate as carbon source

A similar type of the bacterial exopolysaccharide was reported by Moazami (2001) as super water absorbent for the revival of saline and desert lands which acted as water reservoir, salt filter and biofertilizer simultaneously. The studied hydroabsorbent biopolymer also showed the highest capacity of water retention inspite of dry and hot conditions created in the lab, suggesting that the biopolymer acted against water evaporation. Similarly the polymer demonstrated a significant moisture absorption and retention capacity in different relative environmental humidities (hot desert conditions), which was comparable to those of currently used desiccant compounds, i.e. $CaCl_2$, glycerin and silica gel beads, thus it could be used as desiccant. Such type of bacterial exopolysaccharide have also been reported by Ryuichiro and Nohata (1994) which were produced by A*lcaligene latus.* The rheological characteristics of the biopolymer could be used for the stabilization of the soil and to prevent the soil erosion and high rate of evaporation in hot seasons.

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