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Effects of some organic pollutants on the exopolysaccharides (EPSs) produced by some *Pseudomonas* spp. strains

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ABSTRACT

In this study, isolation and characterization of exopolysaccharides produced by *Pseudomonas aeruginosa* B1, *P. fluorescens* B5, *P. stutzeri* B11 and *P. putida* B15 which had been seen to produce exopolymers of potential interest in biotechnological applications were examined. To initiate the observation of the organic pollutants–polymer interactions, the yield and properties of their extracellular polysaccharide were researched. The exopolysaccharide production by these strains during growth in nutrient broth medium (control) was 41–75 mg L^{-1}. Also, *P. aeruginosa* B1, *P. fluorescens* B5, *P. stutzeri* B11 and *P. putida* B15 had exhibited high production of EPSs in presence of various organic pollutants (2,4-D, benzene, BTX and gasoline, respectively) in mineral salt medium (MSM) as a sole carbon source. EPS production by the 4 strains ranged from 40 mg L^{-1} to 8 mg L^{-1}. Monosaccharide composition of EPS produced by these cultures were analyzed by HPLC. Results indicated that EPSs of strains contained neutral sugars and acetylated amino sugars. The neutral sugars in the EPS were mainly composed of glucose, arabinose, glycerol, ribose. The presence of galactronic acid, *N*-acetyl-D-galactosamin and *N*-acetyl-D-glucosamine indicated the acidic nature of the polysaccharide. Glycerol was the basic structural unit of EPS produced by the strains except *P. stutzeri* B11 (MSM with 1% BTX). Strain B1 (in NB medium) was found to be composed of neutral sugars (100%) while strain B1 [in MSM medium with 0.2% (v/v) 2.4-D] contained neutral sugars (70.0%), acetylated amino sugars (30.0%). Also, EPS content of strain B5 (in the NB medium) was neutral sugars (99.8%), acetylated amino sugars (0.2%) while the strain B5 [in MSM medium containing the 1% (v/v) benzene] was found to contain neutral sugars (99.9%), acetylated amino sugars (0.1%). However, EPS monomer composition by strain B11 was detected as neutral sugars (99.77%), acetylated amino sugars (0.23%) in NB medium while the strain B11 [in MSM medium with 1% (v/v) BTX] contained neutral sugars (98.2%) and acetylated amino sugars (1.8%). Lastly, in NB medium by strain B15 was found to contain neutral sugars (99.9%) and acetylated amino sugars (0.1%) while in MSM medium in the presence of 1% (v/v) gasoline it was found to contain neutral sugars (83.6%), acetylated amino sugars (16.4%). Monomer composition of control EPSs changed to different structures in the presence of various organic pollutants. Diversities of organic compounds as carbon source affected the monomer composition of EPS produced by some *Pseudomonas* spp. cultures.

1. Introduction

Exopolysaccharides (EPSs) are produced by many bacteria from clinical and environmental habitats and have an important function in the degradation of toxic pollutants. Recently, the pollution of soil and water by industrial chemicals is a serious problem afflicting the modern world. The use of bioremediation technologies for removing these contaminants provides a safe and economic alternative to commonly used physical–chemical treatment [1–4]. Bacteria are the primary agents of the removal/degradation of environmental toxicants such as herbicides, pesticides, insecticides, various petrol hydrocarbons [5] and they are able to use some of these compounds as the only sources of carbon and energy. Today many microorganisms, specially *Pseudomonas* sp. are used to degrade organic pollutants in order to minimize contamination caused by several industrial activities [6]. However, in recent studies extracellular polysaccharides produced by *Pseudomonas* species have been widely reported [7–10] and polysaccharide production is a common property among these bacteria. Polysaccharides are believed to protect bacterial cells from desiccation, heavy metals, organic compounds or other environmental stresses, including host-immune responses, and to produce biofilms, thus to enhance the chances of the cells to colonize special ecological niches [11–14]. Besides this, the role of polysaccharides in industry is based on their

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performance as speciality chemicals, namely on their capacity to alter the basic properties of water and their propensity for emulsification, suspension, stabilization, floculation, properties which make these polymers suitable for a specific market or application. [15]. Other important applications of microbially produced exopolymers are in the food, pharmaceutical and petroleum industries and in medical fields and they are widely accepted products of biotechnology [16].

In the present paper, it is aimed to investigate the effects of media and various organic pollutants on the yield and properties of EPS produced by *Pseudomonas aeruginosa* B1, *P. fluorescens* B5, *P. stutzeri* B11 and *P. putida* B15 strains.

2. Materials and methods

2.1. Test chemicals

The herbicide, 2,4-dichlorophenoxyacetic acid (CAS RN: 94-75-7, 98% purity) and HPLC-grade solvents were purchased from Sigma. All chemicals used for media preparation and petrol hydrocarbons [Benzene, BTX (Benzene, Toluene, Xylene) and gasoline] were ensured from Merck.

2.2. Bacterial strains

P. aeruginosa B1, *P. fluorescens* B5, *P. stutzeri* B11 and *P. putida* B15 strains used in this research were selected from between 20 cultures exhibited the highest substantial growth in presence of environmental toxicants such as herbicide (2,4-D) and various petrol hydrocarbons (benzene, BTX and gasoline) as a sole carbon source. All the four strains of *Pseudomonas* spp. were obtained from the culture collection of the Biotechnology Laboratory of Gazi University, Department of Biology Faculty of Arts and Science, in TURKEY. All of the strains were stored on Nutrient Agar Medium (Oxoid) slopes at 4 °C and stock cultures were maintained at −20 °C in 0.5% (v/v) glycerol.

2.3. Media and growth conditions

Samples were maintained in 250 mL flasks containing 50 mL of nutrient broth (NB) culture medium [17] (in g/L: peptone 2.5; NaCl 2.5; yeast extract 1.0; beef extract 0.5; pH 7.0) and mineral salt medium (MSM) [17] (in g/L: Na_2HPO_4 4.0; KH_2PO_4 1.5; NH_4Cl 1.0; $MgSO_4{\cdot}7H_2O$ 0.2; $C_6H_8O_7FeNH_3$ 0.05; modified Hoagland trace element solution g/3,6 L: BH_3 11.0; $MnCl_2{\cdot}4H_2O$ 7.0; $AlCl_3$ 1.0; $CoCl_2$ 1.0; $CuCl_2$ 1.0; KI 1.0; $NiCl_2$ 1.0; $ZnCl_2$ 1.0; $BaCl_2$ 0.5; KBr 0.5; LiCl 0.5; Na_2MoO_4 0.5; $SeCl_4$ 0.5; $SnCl_2{\cdot}2H_2O$ 0.5; $NaVO_3{\cdot}H_2O$ 0.1; pH 7.0). NB was used as a growth medium (control) to monitor the growth of EPS production by strains while the MSM containing 1% benzene, BTX, gasoline and 0.2% 2,4-D herbicide was used to determine EPS production as well as the growth (degradation ability) of *Pseudomonas* spp.

1 mL of the active cultures was adjusted to Macfarland 5 for whole assays and inoculated inside the media. The erlanmayer flasks were incubated at 37 °C, by using an incubator shaker (MINITRON) at 100–150 rpm.

NB medium was autoclaved for 15 min at 120 °C but mineral salt solutions were autoclaved separately to prevent precipitation reactions. The organic chemicals were sterilized through 0.45 μm-pore-size type HA membrane filters (Millipore Corp., Bedford, MA).

The experiment was carried out until the culture reached the stationary growth phase (72 h).

2.4. Isolation and quantification of EPS

EPS was extracted by the modified procedure of Cérantola, Bounéry, Segonds, Marty, and Montrozier [18]. Cells were harvested at room temperature by centrifugation at 10,000 × *g* for 10 min. Supernatant was removed. After pellet was dissolved in 1 mL deionized distilled water, it was boiled for 15 min at 100 °C. It was then kept at room temperature for 10 min and added to 3 μL of 85% trichloracetic acid solution (TCA). The mixture was centrifuged at 10,000 × *g* for 30 min. The supernatant which contained EPS was pooled and equal volume of ethanol was added. The mixture was kept at 4 °C overnight and centrifuged at 10,000 × *g* for 30 min again. Precipitate was then washed two times using 95% ethanol and centrifuged at 10,000 × *g* for 30 min. Final precipitate was dissolved in a 1-mL deionized distilled water and stored at −20 °C. Total EPS (expressed as mg per liter) was estimated in each sample by phenol–sulphuric method [19] using glucose as standard [20]. The main values were calculated from the data obtained with duplicate trials.

2.5. Characterization of EPS

The monosaccharide composition of freeze-dried exopolysaccharides samples was determined with HPLC (VARIAN ProStar) by using Metacarb 87H column (300 mm × 7.8 mm, Cat. No. 5210). The organic acids were determined with PDA detector (VARIAN 330) (210 nm), while, the egzopolysaccharides were determined with RI detector (VARIAN 350), connected following to PDA detector. The analyses conditions are; mobile phase 0.008N H_2SO_4, flow rate 0.4 mL min^{-1} and 35 °C. The analyses were accomplished by the Middle East Technical University, Central Laboratory, Molecular Biology and Biotechnology R&D Center (Ankara, Turkey) and studied as two replications.

2.6. Experimental design and statistical analysis

The experiment was performed in a completely randomized fashion with two replicates. Each analysis was done on two samples from each replicate. Results of each representative experiment were analyzed by Pearson correlation (SPSS-11). Person's correlation was used for determine any significant difference between control group of the strains and EPS production amount of the strains exposed to organic pollutants. Significance was determined at $\alpha = 0.01$ level. If significant differences were indicated among treatment means ($P \leq 0.01$), means were differentiated using the least square mean test at $\alpha = 0.01$.

3. Results and discussion

3.1. Isolation and quantitative determination of EPS

In this study, several organic pollutants endangering human health and environment in Turkey were used for EPS production. These organic pollutants, such as 2,4-D, benzene, BTX and gasoline, are preferred as carbon sources for the production of EPS. Hence, firstly, isolation and production of the exopolysaccharides produced by the organic pollutants-degrading bacteria were determined. The composition of the medium plays an important role in the production of EPS [21]. The organic pollutants-degrading bacteria, *P. aeruginosa* B1, *P. fluorescens* B5, *P. stutzeri* B11 and *P. putida* B15 were inoculated in both the NB (as control) and MSM medium containing 2,4-D, benzene, BTX and gasoline. EPS production by these strains ranged from 75 mg L^{-1} to 41 mg L^{-1}. In NB medium, EPS production by *P. aeruginosa* B1 (75 mg L^{-1}), *P. putida* B15 (67 mg L^{-1}), and *P. fluorescens* B5 (63 mg L^{-1}) strains was higher than that of *P. stutzeri* B11 (41 mg L^{-1}) strain. On the other hand, when these cultures were grown in medium containing organic pollutants, the EPS production was 8–40 mg L^{-1} and results were lower than that of NB medium (Table 1). EPS production of the strains exposed to organic pollutants were significantly decreased according to

Table 1
Influence of different carbon sources on EPS production.

Strains	Carbon sources	EPS values (mg L^{-1})[a]
P. aeruginosa B1	Control[b]	75 ± 1.0[c]
	2,4-D[d]	40 ± 0.0[c]
P. fluorescens B5	Control[b]	63 ± 0.0
	Benzene[e]	33 ± 0.0
P. stutzeri B11	Control[b]	41 ± 2.0
	BTX[e]	29 ± 0.0
P. putida B15	Control[b]	67 ± 5.0
	Gasoline[e]	8.0 ± 1.0

[a] Values are means ± standard deviations of triplicate measurements.
[b] It was used nutrient broth medium (v/v) as control.
[c] The highest EPS production in the both media.
[d] It was used minimal salts medium with 0.2% (w/v) concentration.
[e] It was used minimal salts medium with 1% (v/v) concentration.

control group ($P \leq 0.01$). The carbon source used for growth determines both the quality and quantity of polysaccharide formation [22–24]. Similarly, the strain, the culture conditions, and the type of carbon source influence the amount and the composition of microbial EPS that is produced by a certain species [25]. In this study, *P. aeruginosa* B1, *P. fluorescens* B5, *P. stutzeri* B11 and *P. putida* B15 strains showed good EPS production according to control (NB medium) in the presence of various organic pollutants (2,4-D, benzene, BTX and gasoline) in MSM medium as a carbon sources. Similar studies were carried out by Osman et al. [26]; Bonilla et al. [27]. Both of the reported research on EPS production by Pseudomonad (*P. syringae* pv. Glycinea and *P. putida*) has focused on the effect of different culture conditions, and the type of carbon source (sucrose and glucose; ethanol, glycerol and naphthalene) in the production of EPS and this study is supported by both studies reported in the literature. Also, these data suggest that EPS_s may contribute to the protective effects against organic pollutants toxicity.

3.2. Monosaccharide composition of EPS

The monosaccharide compositions of EPS produced by *P. aeruginosa* B1, *P. fluorescens* B5, *P. stutzeri* B11 and *P. putida* B15 were analyzed by HPLC. This study on the monosaccharide composition of polysaccharides was maintained in both NB and MSM medium with organic pollutants. The results showed that EPSs of strains content to neutral sugars and acetylated amino sugars. The neutral sugars in the EPS were mainly composed of glucose, arbinose, glycerol, and ribose. The presence of galactronic acid, *N*-acetyl-D-galactosamin and *N*-acetyl-D-glucosamine indicated the acidic nature of the polysaccharide. The polysaccharides produced by strain B1 in NB medium were composed of glucose (31.0%) and glycerol (69.0%) while in MSM medium with 0.2% (v/v) 2.4-D it was composed of glycerol (70.0%) and *N*-acetyl-D-glucose amine (30.0%). When strain B5 was grown in the NB medium, EPS content was composed of glucose (1.9%), arabinose (20.4%), glycerol (44.7%), ribose (32.8%) and galactronic acid (0.2%) while in MSM medium containing the 1% (v/v) benzene it was composed of glycerol (92.2%), ribose (7.7%) and *N*-acetyl-D-glucose amine (0.1%). On the other hand, EPS monomer composition by strain B11 was detected as glycerol (98.8%), ribose (0.97%), galactronic acid (0.16%) and *N*-acetyl-D-glucose amine (0.07%) in NB medium while in MSM medium with 1% (v/v) BTX it was detected as ribose (98.2%) and *N*-acetyl-D-glucose amine (1.8%). Lastly, in NB medium by strain B15 was found to contain glycerol (99.9%) and *N*-acetyl-D-galactose amine (0.1%) while in MSM medium in the presence of 1% (v/v) gasoline it was found to contain glycerol (83.6%), galactronic acid (16.3%) and *N*-acetyl-D-glucose amine (0.1%) (Table 2). The data obtained showed that monomer composition of control EPSs turned to different structures in the presence of various organic pollutants. Diversities of organic compounds as carbon source affected the monomer composition of EPS produced by *Pseudomonas* spp. cultures. Similarly, several studies have reported that the yield and composition of EPS produced by bacteria can be influenced by the changes in the culture conditions or in the medium composition [28–30]. EPSs containing the neutral sugars have been reported in *Pseudomonas* sp., such as, *P. putida* was found to contain glucose, galactose, and pyruvate in a ratio of 1:1:1 while the *P. fluorescens* polymer contained glucose, galactose, and pyruvate in a ratio of 1:1:0.5, respectively [31]. Some of them also consist of (acetylated) amino sugars [32–34]. Kenne and Lindberg [35] reported that uronic acids, e.g. glucuronic and galacturonic acids, were common components of bacterial extracellular polysaccharides. Yuvali Celik et al. [36] indicated that monomeric sugar compositions of two *Pseudomonas* strains (G1 and G12) in different carbon sources (glucose, mannose, fructose, xylose) were composed of glucose, galactose, mannose, mannitole, glycerol, ribose, and *N*-acetylglucosamine. Bonilla et al., found that *P. putida* ML2 produced the EPS when growing in mineral-defined medium with glucose, glycerol, ethanol and naphthalene as the sole carbon source and its EPS was composed of rhamnose, glucose and glucosamine in a 3:2:1 molar ratio [27]. Royan et al. demonstrated that when grown at room temperature in sodium benzoate as the sole source of carbon, EPS content of *P. mendocina* P_2d cells was composed of rhamnose, fucose, glucose, ribose, arabinose and mannose [16]. Kachlany et al. revealed that the purified EPS from hydrocarbon-degrading *P. putida* G7 contained the monosaccharides, glucose,

Table 2
Monosaccharide composition expressed as percentage (%) of the single monosaccharide on the EPS contents of the control and various organic pollutants of some *Pseudomonas* spp.

Strains	Media	Monosaccharide composition of EPS[a] (%)								
		Glucose	Rhamnose	Mannose	Arabinose	Glycerol	Ribose	Galacturonic acid	*N*-Acetyl-D-galactose amin	*N*-Acetyl-D-glucose amin
P. aeruginosa B1	NB (control)	31.00	–	–	–	69.00	–	–	–	–
	MSM with 0.2% (w/v) 2,4-D	–	–	–	–	70.00	–	–	–	30.00
P. fluorescens B5	NB (Control)	1.90	–	–	20.40	44.70	32.80	0.20	–	–
	MSM with 1% (v/v) benzene	–	–	–	–	92.20	7.70	–	–	0.10
P. stutzeri B11	NB (control)	–	–	–	–	98.80	0.97	0.16	–	0.07
	MSM with 1% (v/v) BTX	–	–	–	–	–	98.20	–	–	1.80
P. putida B15	NB (control)	–	–	–	–	99.90	–	–	0.10	–
	MSM with 1% (v/v) gasoline	–	–	–	–	83.60	–	16.30	–	0.10

–: not detected.
[a] Determined by HPLC.

rhamnose, ribose, *N*-acetylgalactosamine and glucuronic acid [9]. The results reported here demonstrate that the culture conditions and the type of carbon source influence the amount and the composition of EPSs produced by the strains mentioned in this study. The monosaccharides isolated from the strains mentioned in this study were different from other *Pseudomonas* species, but the main sugar components (glucose, ribose, glycerol, galacturonic acids, *N*-acetylglucosamine and *N*-acetylgalactosamine) were similar to those reported by other researchers.

In conclusion, *Pseudomonas* spp. was able to produce the EPS in the presence of various organic pollutants; however, the most efficient carbon source was 2,4-D. The deficiency of the data on the effects of toxic organic pollutants commonly used in Turkey for EPS production and its properties by *Pseudomonas* spp. was made up by the investigation conducted through this study, and the results of the study may have important implications in both the industrial and environmental areas.

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