

# Characterization of Adhesive Exopolysaccharide (EPS) Produced by *Pseudomonas aeruginosa* Under Starvation Conditions

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**Abstract** *Pseudomonas aeruginosa* synthesizes large quantities of exopolysaccharide (EPS), making it an excellent model organism for the study of EPS-mediated adhesion. The purpose of this investigation was to evaluate the influence of limited nutrients availability in the culture medium on the composition of EPS produced by *P. aeruginosa*. The relationship between the EPS production and the adhesion process of the *P. aeruginosa* cells to stainless steel surface (type 316 L) under starvation conditions were also examined. In all experimental variants *P. aeruginosa* produced more EPS with an increase of incubation period upon starvation conditions. Under limited nutrients condition, glucose dominated in the EPS materials. After 6 days of the process, only glucosyl units were detected in the extracellular matrix produced by nutrient-deprived *P. aeruginosa* cells. These extracellular molecules promoted more advanced stages of *P. aeruginosa* biofilm formation on the surface of stainless steel.

## Introduction

A better understanding of bacterial adhesion process is needed for the production of microbiologically safe and good quality products in the food industry. Bacteria colonizing the processing equipment are a potential source of food contamination, which may lead to food spoilage, or transmission of diseases [2, 11]. From a medical

perspective, the attached bacteria on catheters, drains, implants, or lenses can cause serious infections [7]. Despite rigorous cleaning and disinfection procedures, pathogenic and spoilage microorganisms may be isolated from many types of surfaces [11].

Gram et al. [11] suggested that solid materials are often colonized by Gram-negative bacteria that originally exist in aquatic environment (with limited nutrients availability). Among marine bacteria species, *Pseudomonas aeruginosa* is one of the most important food spoilage and human opportunistic bacteria [17]. *P. aeruginosa* may cause off-flavor or the textural changes resulting in sensory rejection of vacuum-packed meats, vegetables, and fish [2]. *P. aeruginosa* cells have also been described as etiological agents in urinary and pulmonary tract infections, as well as in gastroenteritis resulting from the consumption of contaminated food [6, 11].

The ability of *P. aeruginosa* to adhere on surfaces is associated with the extensive production of exopolysaccharide (EPS) [6, 7]. Chen and Stewart [4] concluded that EPS is responsible for both adhesion and cohesion interactions and play a crucial role in maintaining structural integrity of *P. aeruginosa* biofilms. It is also considered that EPS can promote a preconditioning of surface, making the adhesion process more favorable. In some cases, EPS matrix also enables the marine bacteria to capture nutrients [7].

The problem of *P. aeruginosa* attachment process to solid materials has not been solved yet. Most researches have focused so far on the mechanism of EPS secretion and its role in biofilm formation process under optimal nutrients availability in the medium. These cultivation conditions do not correspond to natural environment, where *Pseudomonas* spp. is widely distributed [12]. Reduced nutrient availability in the medium changes the manner of attachment process of cells to solid materials [19]. Moreover, upon conditions of starvation the

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properties of the cell envelope are being modified because of the changes in the monosaccharide composition of EPS [3]. Analysis of the extracellular mechanisms determining the biofilm phenotype on abiotic surfaces will help in the eradication of the attached bacteria [2].

The aim of this study was to define the influence of limited nutrient availability on the monosaccharide composition of EPS produced by *P. aeruginosa* cells. The relationship between the EPS production and the adhesion process of the *P. aeruginosa* cells to stainless steel surface (type 316 L) under starvation condition was also examined. This type of steel is well-suited for making food processing equipments and surgical instruments [18].

## Materials and Methods

### Bacterial Strains and Growth Conditions

*P. aeruginosa* strain ATCC 10145 was obtained from the American Type Culture Collection (Rockville, MD, USA). The strains of *P. aeruginosa* ATCC 10145 commonly thrive in water environment. During the investigations, the microorganisms were passaged three times after every 48 h on the ABPG medium according to Schubert [20]. The ABPG medium contained: 17.0 g/l of peptone from casein, 3.0 g/l of peptone from soy meal, 0.5 g/l of glucose, 5.0 g/l of sodium chloride, 10.0 g/l of L-arginine-monohydrochloride, 0.015 g/l of bromothymol blue, 0.02 g/l of cresol red and 0.0038 g/l of brilliant green. From each passage 10 ml of inoculum of *P. aeruginosa* was added to 100 ml of the fresh medium. The cultures were incubated at 35°C under shaking conditions (100 rpm/min) on the ABPG medium with optimal (as described above) and reduced by 90% (w/v) of optimal nutrients availability. The pH value of the culture medium at the beginning of incubation was 7. The incubation lasted in total 144 h.

### Isolation of Bacterial Exopolysaccharides

Isolation of EPS was based on the procedure employed by Forde and Fitzgerald [8]. The bacteria were harvested by centrifugation at 3000g for 20 min at the room temperature after 24, 48, 72, 96, 120 and 144 h of each experiment. The cells were resuspended in 1.5 ml of 30% (w/v) NaOH. Samples were boiled for 15 min, centrifuged at 15000g for 15 min and the supernatant fluids were added dropwise to 60% (v/v) ethanol.

### Quantification of EPS

The total EPS (expressed as µg/CFU) was determined using the acid hydrolysis method of Parkar et al. [16]. The

precipitated EPS was collected by centrifugation (15000g, 20 min) and was resuspended in 1 ml of sterile water. The samples were mixed with 7 ml of 77% (v/v) H<sub>2</sub>SO<sub>4</sub> and cooled for 10 min in an ice-bath. 1 ml of 1% (w/v) of cold tryptophan was added and the samples were heated in a boiling bath for 20 min to effect hydrolysis. The acid hydrolysis of EPS produced a furan which condenses with the tryptophan and forms a colored product. This was evaluated after cooling the samples by measuring O.D.<sub>500</sub>. Calibration curves were prepared against standard dextran (Mp. 40000) solutions (Sigma, USA).

### Determination of the EPS Monomer Composition

The method described by Forde and Fitzgerald [8] was used for the preparation of EPS for chromatographic analysis. One milli liter of sample was hydrolysed with an equivalent volume of 10% (v/v) H<sub>2</sub>SO<sub>4</sub> for 60 min at 100°C. After cooling, trichloroacetic acid (TCA) was added to a final concentration of 5% (w/v) to precipitate proteins, followed by centrifugation at 15,000g for 20 min at 4°C. The samples were passed through 0.22 µm Millex-GS filters (Milipore, USA). Determination of EPS components (glucose, fructose, galactose, arabinose) was carried out using HPLC technique, on MERCK-HITACHI system with autosampler (model L-7250), pump (model L-7100) and refractive index detector (model L-7490). Analysis were performed isocratically at a flow rate of 0.6 ml/min (0.005 M H<sub>2</sub>SO<sub>4</sub>), at 35°C, on column Aminex HPX-87H, 300 × 7.8 mm (Bio-Rad, USA). Standards were used to identify peaks in chromatograms. Peak areas were used to determine the samples concentration. It was carried out by computer integration (Chromatography Data Station Software, MERCK-HITACHI) operated in the mode of external standard. Standard calibration solutions for examined sugars were prepared within the range 0.1–0.01 mg/ml.

### Stainless Steel Plates Preparation

Stainless steel plates (type 316 L) sized 1 cm × 6.5 cm × 1 mm were treated with 50% (v/v) solution of HNO<sub>3</sub> for 10 min at 70°C. After soaking under distilled water, the plates were put into glass containers and sterilized at 121°C for 15 min [16].

### Bacterial Adhesion Analysis

Bacterial adhesion analysis was started after 144 h of experiments. The stainless steel surface was put into *P. aeruginosa* cultures resulting from the final passage in ABGP medium. At 145 h the plates were removed from glass containers and washed with PBS solutions (pH 7.2) in

order to remove unattached cells from the surfaces. The plates were stained with 0.01% (w/v) solution of acridine orange (2 min). Stained adherent bacteria were observed under oil immersion, using a fluorescence microscope (CARL-ZEISS, Axiovert 200, Germany). To determine the level of *P. aeruginosa* adhesion to the surface of stainless steel the method described by Le Thi et al. [15] was carried out. This method is based on the direct classification by the operator of randomly selected 50 visual fields according to a 9-degree scale:

- 1st degree: from 0 to 5 bacteria cells in visual field.
- 2nd degree: from 5 to 50 bacteria cells in visual field.
- 3rd degree: only single bacterial cells (above 50 bacterial cells in visual field; no microcolonies).
- 4th degree: single bacterial cell + small microcolonies.
- 5th degree: large but not confluent microcolonies + single bacterial cell.
- 6th degree: confluent microcolonies + single bacterial cell.
- 7th degree: 1/4 visual field covered by the biofilm.
- 8th degree: 1/2 visual field covered by the biofilm.
- 9th degree: visual field totally covered by the biofilm.

The classification procedure excluded bacteria situated on the edges of each visual field.

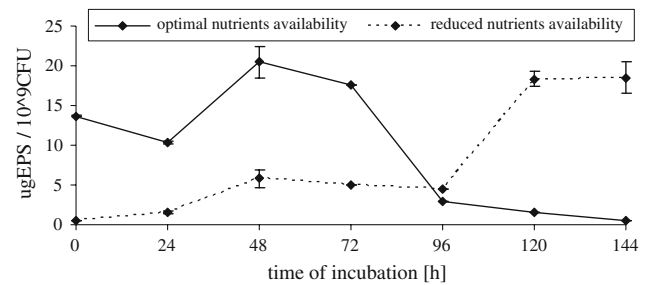
#### Statistical Analysis

Presented results are the average of three independent experiments. Student's *t*-test was used to determine the significant difference ( $P < 0.05$ ) between the carbohydrate compositions of EPS produced by cells under different nutrients availability in the medium.

## Results

#### Quantitative Determination of EPS

To characterize the physiological potential of *P. aeruginosa* cells for colonization of the abiotic surfaces under starvation conditions, the quantitative determination of EPS was carried out. The EPS production capacity of *P. aeruginosa* at different nutrients availability in the medium is shown in Fig. 1. *P. aeruginosa* produced more EPS with an increased incubation period upon starvation conditions. In the first 96 h of the process, the EPS production by examined bacteria was not higher than  $5 \mu\text{g}/10^9 \text{CFU}$ . At 120 h of cultivation the EPS synthesis was significantly increased to the level of  $18 \mu\text{g}/10^9 \text{CFU}$ . From 120 h of the experiment the EPS secretion by *P. aeruginosa* remained relatively constant. In contrast, upon favorable conditions the maximum EPS production



**Fig. 1** EPS production by *P. aeruginosa* under different nutrients availability in the medium (Error bars represent standard deviation)

by *P. aeruginosa* ( $20 \mu\text{g}/10^9 \text{CFU}$ ) was observed in 48 h of the process. After 72 h the EPS synthesis decreased to the level of  $3 \mu\text{g}/10^9 \text{CFU}$ .

#### Composition of EPS

Determination of the EPS monomer composition was carried out after acid hydrolysis method. The results of the experiments were expressed as the percentage of examined components in EPS material measured by the spectrophotometric method. Table 1 presents the influence of nutrients availability in the medium on the monosaccharide compositions of EPS produced by *P. aeruginosa* cells. Under conditions of starvation, glucose dominated in the EPS materials. From 120 h of experiments, the content of glucose in the EPS materials increased to the level of 41%. Interestingly, at 144 h of the process, only glucosyl units were detected in the extracellular matrix. In all experimental variants, the percentage of fructose, galactose, and arabinose in extracellular materials was not higher than 4%. Upon nutrient-rich conditions, *P. aeruginosa* synthesized the EPS composed of glucose, fructose, galactose, and arabinose. However, the contents of sugars in the extracellular matrix equaled between 3 and 7%. At 144 h of cultivation under optimal nutrients availability, the content of glucose in the EPS materials significantly increased to the level of 26%.

#### Adhesion

The bacterial colonization of solid material is a multi-step process. In this work to define the rate of biofilm development process, the 9-degree scale according to Le Thi et al. [15] was used.

The results of the influence of nutrients availability on the attachment of *P. aeruginosa* cells to stainless steel (type 316 L) are presented in Table 2. In this work, the adhesion analysis started when *P. aeruginosa* produced the high amount of EPS composed of glucose under starvation conditions. Approximately  $10^7$ – $10^8 \text{CFU}/\text{ml}$  *P. aeruginosa* cells were present in the culture medium during the experiments.

**Table 1** Carbohydrate compositions of EPS produced by *P. aeruginosa* under different nutrients availability in the medium

Time of incubation (h)	Nutrients availability in the culture medium									
	Composition of the EPS material (%)									
	Optimal					Reduced				
	Glucose	Fructose	Galactose	Arabinose	Other	Glucose	Fructose	Galactose	Arabinose	Other
24	1.10 <sup>a</sup> (±0.06)	0.59 <sup>a</sup> (±0.05)	0.32 <sup>a</sup> (±0.05)	0.01 <sup>a</sup> (±0.001)	97.57 <sup>a</sup> (±1.7)	8.45 <sup>a</sup> (±0.04)	10.14 <sup>a</sup> (±0.23)	3.04 <sup>a</sup> (±0.61)	1.52 <sup>a</sup> (±0.05)	76.84 <sup>a</sup> (±0.2)
48	4.98 <sup>b</sup> (±0.15)	0.22 <sup>b</sup> (±0.07)	0.28 <sup>a</sup> (±0.05)	0.03 <sup>b</sup> (±0.01)	94.49 <sup>a</sup> (±1.5)	17.29 <sup>b</sup> (±1.3)	2.16 <sup>b</sup> (±0.05)	3.89 <sup>a</sup> (±0.72)	1.74 <sup>a</sup> (±0.3)	74.92 <sup>a</sup> (±1.78)
72	0.70 <sup>c</sup> (±0.05)	0.48 <sup>a</sup> (±0.08)	0.15 <sup>b</sup> (±0.04)	–	98.67 <sup>a</sup> (±2.2)	21.62 <sup>b</sup> (3.5)	7.35 <sup>c</sup> (±1.34)	–	–	71.03 <sup>b</sup> (±0.83)
96	3.48 <sup>d</sup> (±0.01)	2.96 <sup>c</sup> (±0.7)	0.70 <sup>c</sup> (0.01)	0.17 <sup>c</sup> (±0.05)	92.69 <sup>a</sup> (±4.3)	17.40 <sup>b</sup> (5.13)	0.73 <sup>d</sup> (±0.05)	0.68 <sup>b</sup> (±0.09)	–	81.19 <sup>c</sup> (±0.1)
120	6.60 <sup>e</sup> (±0.06)	6.60 <sup>a</sup> (±1.6)	1.32 <sup>d</sup> (±0.07)	0.34 <sup>d</sup> (±0.1)	85.14 <sup>b</sup> (1.1)	44.63 <sup>c</sup> (±5.23)	0.72 <sup>d</sup> (±0.03)	0.72 <sup>b</sup> (±0.05)	–	53.93 <sup>d</sup> (±0.8)
144	26.37 <sup>f</sup> (±3.5)	6.33 <sup>a</sup> (±0.82)	3.37 <sup>e</sup> (±0.4)	1.37 <sup>e</sup> (±0.51)	62.56 <sup>c</sup> (±1.54)	41.29 <sup>c</sup> (±3.51)	–	–	–	58.71 <sup>e</sup> (±1.3)

– Not detected

Average values ± standard deviations

Values followed by different letters in columns differ significantly at the  $P < 0.05$  level**Table 2** The influence of nutrients availability on *P. aeruginosa* adhesion to stainless steel surface (type 316 L)

Time of incubation (h)	Nutrients availability in the culture medium					
	Optimal			Reduced		
	Cell number in the medium (CFU/ml)	Dominant adhesion degrees	Appearance of higher adhesion degrees (6,7,8,9)	Cell number in the medium (CFU/ml)	Dominant adhesion degrees	Appearance of higher adhesion degrees (6,7,8,9)
145	$6.0 \times 10^8$	4	–	$4.8 \times 10^7$	6, 4	6, 7

– Not detected

In this work when a particular degree of adhesion occurred with a minimum amount of 20% that degree became the dominant one. Stainless steel type 316 L was efficiently colonized by *P. aeruginosa* under starvation conditions. Limited nutrients availability induced more developed stages of *P. aeruginosa* biofilm formation process on the surface of stainless steel (6 and 7th adhesions degree). *P. aeruginosa* grown upon nutritionally favorable conditions colonized the studied surface at the 4th degree of adhesion and no higher level of adhesion (6–9th degrees) was observed in the work.

## Discussion

In this work, we aimed to characterize the EPS synthesis by *P. aeruginosa* cells during growth on low nutrients availability in the medium. The work of Wai et al. [21]

concluded that bacterial EPS production is favored by nutrients deprivation. However, in the literature there are not clear evidences about the mechanisms of that process. Nutrient limited conditions represent the natural aquatic habitat of *P. aeruginosa* cells [12].

In the food industry and hospital conditions, EPS-rich strains are difficult to overcome [2]. The EPS surrounding the microorganisms restrict disinfectant and antibiotic penetration [10]. The extensive production of EPS by microorganisms may seriously affect the quality and safety of the processed food and pose a potential risk to patients [7]. In our study, long-term starvation influenced higher productivity of EPS matrix by *P. aeruginosa* cells. The highest yield of EPS production by examined bacteria was observed after 120 h of cultivation. Kiliç and Dönmez [14] noticed similar effects when monitoring EPS production by *P. aeruginosa* cells. According to this study, EPS synthesis by *P. aeruginosa* was effected by the increasing incubation

period. The highest production of extracellular matrix by examined cells was noticed after incubation for 96 h. Dunne [7] reported that extensive production of EPS by nutrient-deprived bacteria is a logical strategy of cells. The EPS matrix is an efficient system for trapping nutrients from surrounding environments [7].

In this work, monosaccharide compositions of EPS matrix produced by *P. aeruginosa* upon low nutrients availability in the medium were also investigated. This knowledge may improve the elimination the particular pathogenic and spoilage promoting compounds [11]. However, in the literature the qualitative data on the composition of EPS produced by marine bacteria upon starvation are lacking. Our results indicated that glucose dominated in the EPS material produced by *P. aeruginosa* under starvation conditions. In contrast, Hung et al. [13] concluded that galactose, mannose, and arabinose were the main compositions of EPS produced by *Pseudomonas fluorescens* Biovar II. According to this data the content of glucose in EPS material synthesized by examined bacteria was not higher than 5%.

In our study, at 144 h under starvation conditions only glucosyl units were detected in the EPS matrix. Our results underscore the genetic analysis of extracellular material produced by the strains of *P. aeruginosa* [9]. These investigations demonstrated that the synthesis of a glucose-rich polymer is the feature of *P. aeruginosa* strains. These results differ from those published by Grobe et al. [12] where they reported that *P. aeruginosa* synthesized large quantities of extracellular alginate.

In this work, the relationship between the EPS production and the adhesion process of *P. aeruginosa* cells to stainless steel type 316 L upon starvation were also investigated. Microbial attachment process to abiotic surfaces is determined by EPS production [7]. In our study, the attachment analysis of *P. aeruginosa* cells started when examined bacteria producing EPS matrix composed of glucose upon starvation conditions. According to the results published by Costerton et al. [5], we assumed that this extracellular polysaccharide may determine the biofilm phenotype upon starvation conditions. The researchers prepared antibodies against a planktonically synthesized polymer and used them to reveal interaction with material in the biofilm matrix. This indicated that some components of the biofilm EPS had the same compositions as the planktonic product. In this work, starvation conditions increased the adhesion of microorganisms to abiotic surfaces. Our studies also indicated that high quantities of EPS are not required for the first step in biofilm formation but are needed to develop a true biofilm matrix. Similar results were observed for a wild type of *P. fluorescens* and a nonpolysaccharide-producing mutant [1]. Both strains adhered to a glass surface, but over time, the wild bacteria

formed three-dimensional structure while the mutant remained as single adherent cells on the surface.

Our data support the notion that the composition of adhesive EPS produced by *P. aeruginosa* cells should be examined under conditions in which marine bacteria are widely distributed. The production of EPS molecules by examined microorganisms is more extensive upon long-term starvation. In response to nutrient limitations in the medium *P. aeruginosa* synthesized EPS composed of glucose. These extracellular molecules promoted more advanced stages of *P. aeruginosa* biofilm formation on the surface of stainless steel upon starvation conditions.

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