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Polysaccharide production and biofilm formation by *Pseudomonas fluorescens*: effects of pH and surface material

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Abstract

Although the synthesis of extracellular polysaccharides was first recognized in certain bacterial cultures a long time ago, its role in bacterial adhesion is still subject to some debate.

Several fermentation batch cultures were performed under different conditions of pH (pH 7, maintained with NaOH and HCl; pH 7 in phosphate buffer, and without pH control) in order to study the relation between the production of extracellular polysaccharides and biofilm formation on polymeric slides suspended in the culture medium. The polymers used were polystyrene, polypropylene, polyethylene and poly(vinyl chloride).

The maximum amount of exopolysaccharides in the culture medium occurs at pH 7, although slightly thicker biofilms seem to be formed when there is no pH control.

The biofilms were analysed by scanning electron microscopy and by wavelength dispersion spectroscopy. Biofilm morphology seems to be much more dependent on the type of substratium than on the pH of the medium; for different pH values, a polymeric network can be more clearly observed on biofilms formed on all surfaces except poly(vinyl chloride).

Key words: Biofilm formation; pH; Polymer surfaces; Polysaccharides; *Pseudomonas fluorescens*

Introduction

Several microorganisms, especially bacteria, are capable of producing extracellular polysaccharides in a variety of environments. It has been suggested that the production of exopolysaccharides (EPSs) is a direct and logical response to selective pressures in ecological niches. This means that the main function ascribed to EPSs is of a protective nature.

There are some environments where the survival of microorganisms depends on their ability to adhere to a surface. It seems that EPS-producing strains are more likely to adhere and to develop as thick biofilms. This leads to a generalized idea that the EPSs can have an important role in the adhesion process. However, there is still some debate about the extent of the involvement of EPSs in the first stage of adhesion.

Some authors consider that EPSs can promote a preconditioning of the surface, making adhesion more favourable from a thermodynamic point of view, which means that the change in the free energy of adhesion (ΔF^{ad}) becomes more negative. Others assume that different cellular factors, like external appendages (e.g. pili or fimbriae) can be more determinant for the sticking ability, while still recognizing the benefits of EPSs in subsequent events, namely the stabilization and persistence of the colonies [1].

The aim of this work was to study the production of EPSs as a function of the pH of the medium in

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order to determine if there was any relation between EPS production and the formation of biofilms on polymeric surfaces. The selected microorganisms was the bacterium *Pseudomonas fluorescens*, on account of its strong EPS production capacity and its lack of pathogeny.

Experimental

Microorganism and batch fermentations

The strain used in these experiments was *Pseudomonas fluorescens* isolated from a river water, obtained from the culture collection of the Microbiology Department of the Calouste Gulbenkian Foundation, Oeiras, Portugal. The batch cultures were performed in two identical 2 l aerated and agitated glass fermenters, with facilities for the automatic control of temperature and pH. *Pseudomonas fluorescens* was grown at 27 °C in a medium containing 5 g l^{-1} of glucose, 2.5 g l^{-1} of peptone and 1.25 g l^{-1} of yeast extract under the following conditions of pH: pH 7, controlled with NaOH and HCL; pH 7 in phosphate buffer (0.2 M Na_2HPO_4–0.2 M NaH_2PO_4) and without pH control. For every case the procedure was repeated four times.

Biomass determination

The increase in biomass during the growth process was determined by measuring the optical density (OD) of the fermentation liquor at 660 nm, the samples being collected around every 3 h. Biomass concentration (g l^{-1}) was determined by means of a calibration curve.

Estimation of polysaccharides in the broth

Part of each sample, collected for biomass determination, was centrifuged and filtered through a Whatman membrane of 0.2 μm. A 10 ml portion of cold ethanol (−10 °C) was added to 5 ml of the filtrate and kept for 48 h at 5 °C to allow polysaccharide precipitation. The suspensions were then agitated in a vortex and their OD were read at 280 nm. A small portion of the filtrate (100 μl) was assayed for proteins using the Coomassie Brilliant Blue G250 method as proposed by Bradford [2]. Polysaccharides were identified using the phenol–sulfuric acid colorimetric method of Dubois et al. [3]. Another 20 μl of the filtrate were used to determine the glucose concentration by means of a Sera-Pak kit from Miles Italiana, based on the glucose oxidase method.

Biofilm formation assay

One millimetre thick slides (5 cm × 3 cm) of the polymeric materials polystyrene, polypropylene, polyethylene and poly(vinyl chloride) (PVC) were used as substrata for biofilm development. The polymeric slides were thoroughly washed with a detergent and rinsed with distilled water. Sets of four plates, one of each type of polymer, were displayed like the teeth of a comb (by means of a plastic-covered wire) and immersed in ethanol. On account of their nature the slides could not stand high temperatures, so they were removed from ethanol and suspended in the culture medium, aseptically, after the fermenter had been autoclaved. At the end of the fermentation, the slides were removed, dried in a stove at 70 °C and observed in a scanning electron microscope (Jeol SM 35), coupled to a wavelength dispersion spectroscopy (WDS) system.

Results

Biomass production

The growth curves obtained from each experiment enabled the determination of the specific growth rate μ and the time of duplication, t_d. The average values for each type of fermentation are shown in Table 1.

EPS production

Owing to practical difficulties in obtaining isolated dried polysaccharides it was not possible to

Table 1
Average values for specific growth rate μ, time of duplication t_d, end of exponential growth phase Θ, optical density (OD) of polysaccharides and biomass concentration X (the last two at the end of the exponential phase), for the three different types of fermentation

Type of fermentation	μ (h^{-1})	t_d (h)	Θ (h)	OD	X ($g\ l^{-1}$)
Without pH control	0.48	1.60	10.0	0.077	0.18
pH 7, control with NaOH/HCl	0.65	1.00	8.4	0.098	0.53
In phosphate buffer, pH 7	0.69	0.98	8.4	0.100	0.67

draw the calibration curve of OD (absorbance) vs. concentration. The formation of EPSs was exclusively observed through the rise in the OD.

Proteins are also precipitable by ethanol, so the filtrate obtained from the fermentation liquor was assayed by the method of Bradford [2], as mentioned before, but no detectable amounts of protein were found.

In every type of fermentation the production of EPSs started with cell growth and increased in the course of the fermentation. There was no decrease in the EPS amount, even when the cell density began to decline (see for example Fig. 1). In order to compare EPS production in the different types of culture, the standard considered was the end of the exponential phase, since the experiments did not all have the same duration. Table 1 presents the average values of the time corresponding to the end of the exponential phase.

In the fermentations without pH control and in phosphate-buffered medium a decrease in pH occurs together with a drop in glucose concentration, but when this concentration is around $0.3\ g\ l^{-1}$ the pH rises again. In the experiments of long duration, the final pH attained values even higher than the initial value. Figure 1 shows an example of this variation.

Biofilm formation

The observation of the polymeric slides by scanning electron microscopy showed that they were all completely covered with a deposit on both sides. The deposits seem to be slightly thicker in the experiments performed without pH control.

The morphology of the biofilms is very similar for those formed on the polypropylene, polystyrene and polyethylene slides. Actually, these deposits have the aspect of a film and it is quite impossible to distinguish the shape of the cells, since they are linked and covered by a large amount of polysaccharides. When dried, these films break in several places, as can be seen in Fig. 2.

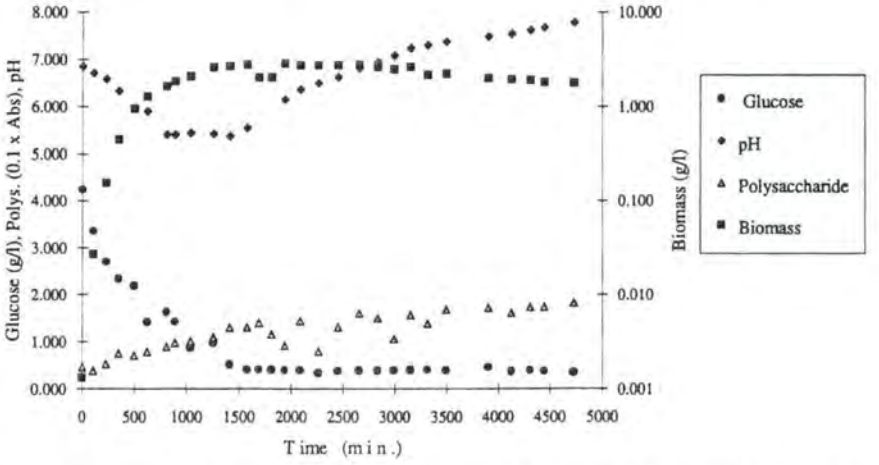

Fig. 1. Experimental values obtained during a fermentation performed in phosphate buffer medium.

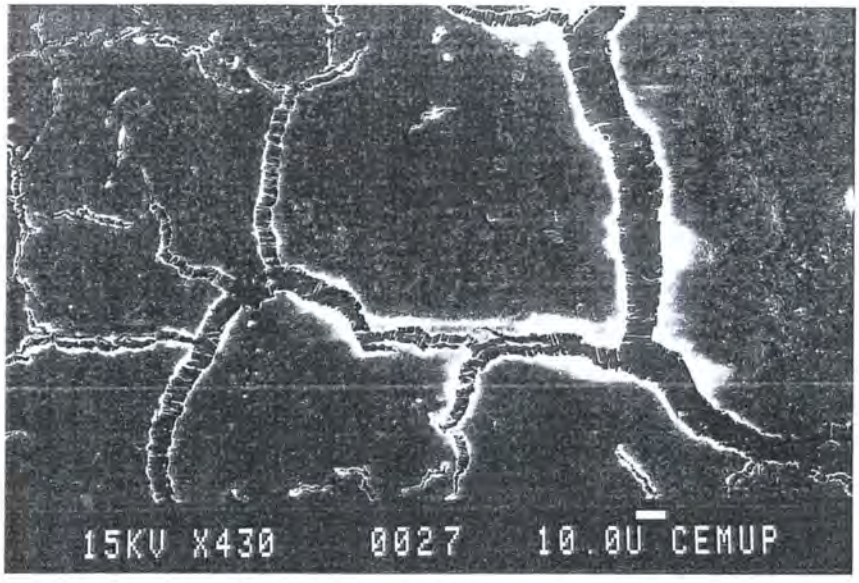

Fig. 2. Micrograph of a biofilm formed on a polyethylene slide during a fermentation performed without pH control.

In the case of the deposits formed on the PVC slides, the polymeric network is not so developed and in most cases it is possible to see the individual cells, although there are a great many of them covering the surface (Fig. 3).

It must be said that at first sight the deposits formed at pH 7 (controlled with NaOH and HCl) have a very peculiar aspect, because a dendritic network can be seen on top of the biofilm (Fig. 4). When analysed by WDS this network proved to be formed by NaCl crystals. Nevertheless, when the observation is directed to the space between

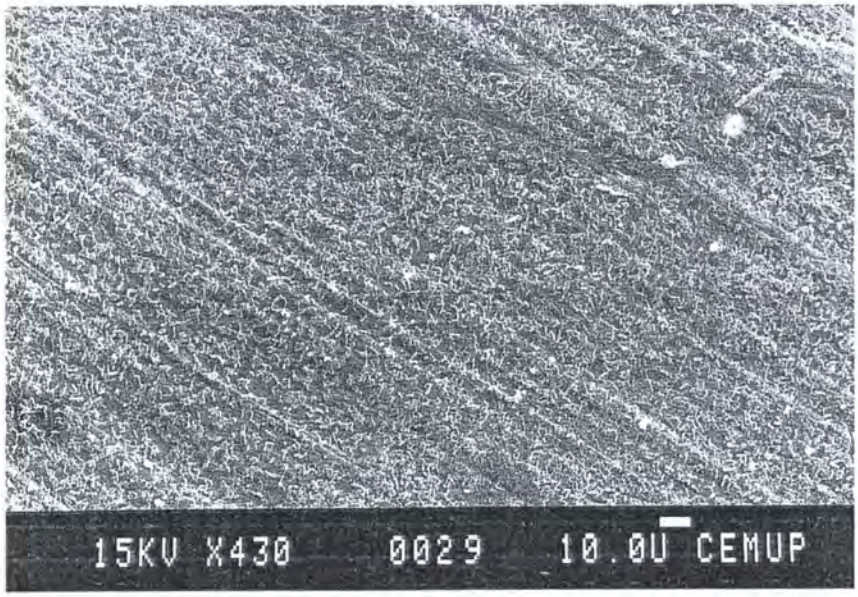

Fig. 3. Micrograph of a biofilm formed on a PVC slide during a fermentation without pH control.

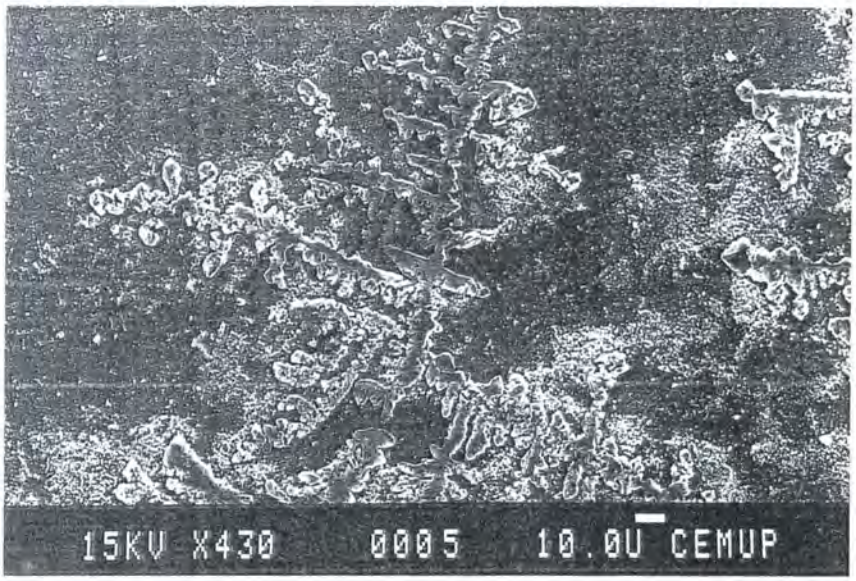

Fig. 4. Micrograph of a biofilm formed on a PVC slide during a fermentation performed at pH 7, the pH being controlled with NaOH and HCl.

dendritic branches, the morphology is that mentioned above. The formation of this network could have occurred during the drying process, on account of the Na^+ and Cl^- ions present in the liquid medium.

Discussion

The amount of EPS produced by this strain of *Pseudomonas fluorescens* appears to be favoured when the initial pH of the medium is 7, the same being valid for biomass production. According to Wilkinson [4], the optimum pH for EPS production depends on the individual species, but it is near neutrality for most bacteria. Several authors [5,6] found that at low phosphate concentrations polysaccharide production is reduced. They assumed that this was due to a decrease in the buffer capacity of the medium so that during the course of the fermentation the pH fell to a point at which the microorganisms suppressed EPS formation. However, the response of the strain under consideration to an acidic pH is quite different, the strain behaving like *Pseudomonas* sp. strain EPS-5028 [7] and *Xanthomonas campestris* [8], which synthesize EPSs throughout the fermentation. In the present case, EPS production occurs together with growth and continues maximally during the stationary phase.

The polysaccharide concentration in the medium does not seem to have a direct relationship to the thickness of the biofilms formed on the polymeric slides. In other words, the microorganisms adhere to the surface, and probably the production of polysaccharides is a response to the surface material. Another hypothesis is that the composition of the surface can accelerate or delay the EPS production. These speculations arise on account of the morphology of the deposits formed on PVC slides. In Fig. 4, in the regions free of NaCl dendrites, it is possible to see that the deposit (formed at pH 7) has a granular structure like the deposit represented in Fig. 3 (formed without pH control). Observations at a higher magnification (× 5000) clearly showed the cells with well-defined contours. If the PVC surfaces were not so extensively covered by cells it could be thought that adhesion was not thermodynamically favoured owing to the surface free energies of the materials present. According to the surface free energy values, determined by

Busscher et al. [9] for the polymeric materials in question, PVC is neither the most nor the least favourable. From this point of view, polystyrene would be expected to be the worst.

It is hoped that this work may open some trails for further studies towards a better understanding of biofilm formation.

Acknowledgement

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