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ROLE OF THE POLYMERIC MATRIX CONSTITUENTS ON THE PERFORMANCE OF A BIOCIDE AGAINST *Pseudomonas fluorescens* BIOFILMS

M. O. Pereira, M. J. Vieira

Centro de Engenharia Biológica-IBQF, Universidade do Minho, 4710-057 Braga, Portugal

ABSTRACT

The effectiveness of glutaraldehyde – a very common biocide – to control biofilms formed by *Pseudomonas fluorescens* on stainless steel slides, was investigated. The tests were performed using two concentrations of the biocide (50 and 100 mg L^{-1}), biofilms of two ages (7 and 15 days), several pH values (5, 7 and 9) and a range of exposure times (from 0 to 1, 3, 7 and 24 hours). The GTA action on biofilm and planktonic populations was assessed by means of activity tests and wet weight of the biofilms. The results showed that biofilms were not completely removed after the treatment with biocide, in all the situations studied. The higher concentration was more effective in reducing the bacterial activity of the biofilm. The biocide proved to be more effective for longer exposure times. GTA showed good antimicrobial activity against P. fluorescens in suspension, with higher activity for pH 9. The findings of this study suggest that when GTA is used to control biofilms, it reacts with one of the components of the matrix – the proteins – thereby reducing its antimicrobial action.

KEYWORDS

Glutaraldehyde; biofilm characteristics; *Pseudomonas fluorescens*; polymeric matrix.

INTRODUCTION

The unwanted accumulation of biofilms in industrial equipment – biofouling – is a natural occurrence, due to the favorable conditions. Biocides still represent the more significant countermeasure to control biofouling formation. However, these chemical substances are not fully effective to remove the biofilm. On that account, to increase the biocide concentration applied or to change to another more aggressive are the more frequent practices to overcome the problem. It is of major importance to know that, when a biocide is applied, the microbial response to the agent will depend not only on the type of microorganisms and type of chemical agent (Morton *et al.*, 1998) but also on the complex interactions established between the biocide and the biofilm matrix.

Glutaraldehyde (GTA) is one of the biocides widely used in industry and in hospital environments (Bott, 1995; Walsh *et al*., 1999). Its biocidal effect is attributed to its two aldehyde groups, that interact with microbial cell constituents, reacting with ammonia and primary amines and more slowly with secondary amines, binding strongly to outer cellular layers (Eagar *et al*., 1986; Cloete *et al*., 1998). Thus, the cell will be less capable of performing its essential cellular functions (Russel, 1994).

The aim of this work was to clarify the way of action of the glutaraldehyde (GTA) on biofilms formed by *Pseudomonas fluorescens*, an abundant bacteria in biofilms formed in industrial equipment, concerning the possible interactions between the biocide and the components of the biofilm matrix.. The experimental tests were performed using two concentrations of GTA (50 and 100 mg L^{-1}), biofilms of two ages (7 and 15 days) and a range of exposure times. Tests were also performed with *P. fluorescens* suspensions for comparison purposes.

MATERIAL AND METHODS

Microorganism: *Pseudomonas fluorescens*ATCC 13525 was used throughout this work.

Biocide: A non-oxidising biocide solution composed of glutaraldehyde (50% w/v) in water was tested.

Experiments with suspended microorganisms:

Microorganism growth: A continuous pure culture of the *P. fluorescens* bacteria was as described elsewhere (Pereira *et al*. 1998)

Biocide treatment: Periodically, a suitable amount of *P. fluorescens* culture was removed from the fermenter, centrifuged (3777g, 10 min) and washed three times with phosphate buffer. The pellets were ressuspended in one of the following buffers: phosphate buffer pH 7 and pH 9 and acetate buffer pH 5, according to the pH value wanted in each assay. The bacterial culture was then divided by several sterilised glass flasks and put in an orbital shaker (120 rpm). After 30 min, a known amount of biocide was added to each flask in order to obtain 50 or 100 mg L^{-1} of GTA.
Determination of the cellular activity: At known time intervals (immediately after biocide addition, and after 1, 3 and 7 h), a sample of each flask was transferred into a respiration chamber to evaluate their cellular activity (through oxygen consumption).

Experiments with biofilms:
Biofilm set-up: Biofilms were formed on stainless steel slides (2 cm x 2 cm and 1 mm thick) that were placed within a well stirred reactor containing a continuous bacterial suspension. The fermenter was fed with a sterile nutrient solution consisting of 40 mg glucose L^{-1}, 20 mg peptone L^{-1} and 10 mg yeast extract L^{-1}, in phosphate buffer pH 7.
Biocide treatment: After biofilm development (7 or 15 days) the biofilm covered metal slides were carefully transferred to a closed vase that contained the biocide solution (50 or 100 mg L^{-1} of GTA). At known time intervals (immediately after the GTA addition, 1, 3, 7 and 24 h of biocide contact time) the steel slides *plus* biofilm were carefully removed from the biocide-containing flask and reserved for evaluation of the GTA action.
Biofilm mass quantification: Before being inserted in the fermenter, and after been degreased, rinsed and dried, the metal slides were identified and weighed. After 7 or 15 days of biofilm formation, and as soon as they were removed, the wet mass of the slides *plus* attached biofilm was also determined. After the GTA treatment, the slides were weighed again. Biofilm mass accumulated on the several slides was thus calculated as the difference between the two respective weights and expressed in kg *per* m^2 of surface area of the slide.
Scrapping and disaggregation of the biofilms: The biofilm that covered the metal slides was completely scraped off the metal and ressuspended into 10 mL of phosphate buffer pH 7. The homogenised suspensions of biofilms were used to assess the cellular activity of the biofilm. The biofilms suspensions that were not treated with GTA were also used to determine the total and extracellular macromolecular composition.
Extraction procedure: The extraction of the extracellular components of the biofilm was carried out using a Dowex resin (50X8, Na^+ form, 20-50 mesh, Aldrich-Fluka 44445) according to the procedure described by Jahn and Nielsen (1995).
Chemical analysis of the biofilm: The chemical analyses were carried out on the homogenised biofilm suspensions, before biocide treatment, and before and after the extraction procedure. The proteins were determined using the Lowry modified method (SIGMA-Protein Assay Kit nº P5656) and the polysaccharides by the phenol-sulfuric acid method of Dubois *et al.* (1956).

Oxygen Uptake Rates Measurement: The respiratory activity of the several samples was evaluated by measuring oxygen uptake rates in a biological oxygen monitor (BOM) in short-term assays. The assays were performed in a Yellow Springs Instruments BOM (Model 53) and the procedure used was described elsewhere (Pereira *et al.*, 1998).

RESULTS

The GTA action on biofilms formed on the metal slides was studied by the assessment of the variation of the mass of the deposit experienced during the treatment period, the determination of the respiratory activity after the GTA treatment and through the evaluation of the chemical analysis of the deposit.The results obtained demonstrated that: i) biofilms were not completely removed after the GTA treatment, in all the situations studied (Figure 1); ii) the higher GTA concentration was more effective in reducing the bacterial activity of the biofilm (Figure 2); iii) the biocide proved to be more effective for longer exposure times; iv) GTA showed good antimicrobial activity against *P. fluorescens* in suspension, with higher activity for pH 9 (Figure 3); v) the macroscopic composition of a *P. fluorescens* biofilm (Table

1) shows that the polymeric matrix of the biofilm had a high content of proteins, additionally to the proteins located at the bacterial cell surface.

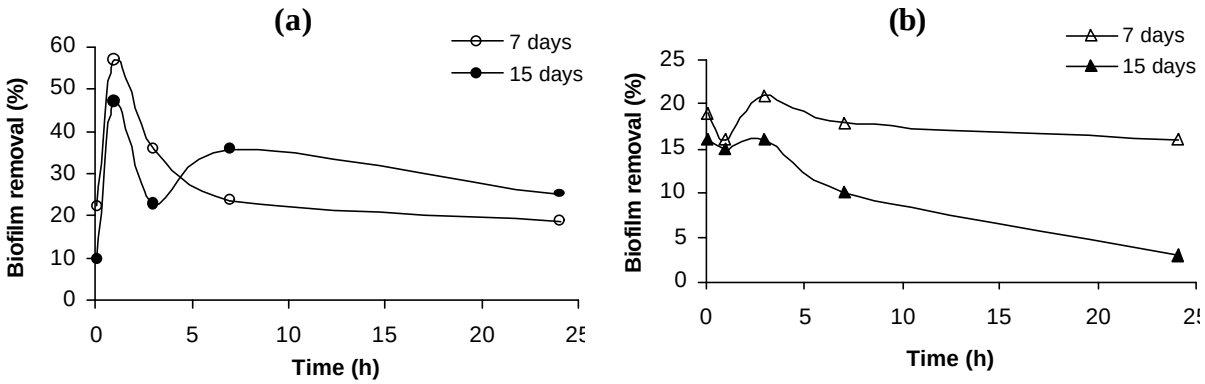

Figure 1. Biofilm removal after application of 50 (a) and 100 mg L^{-1} (b) of GTA, as a function of time.

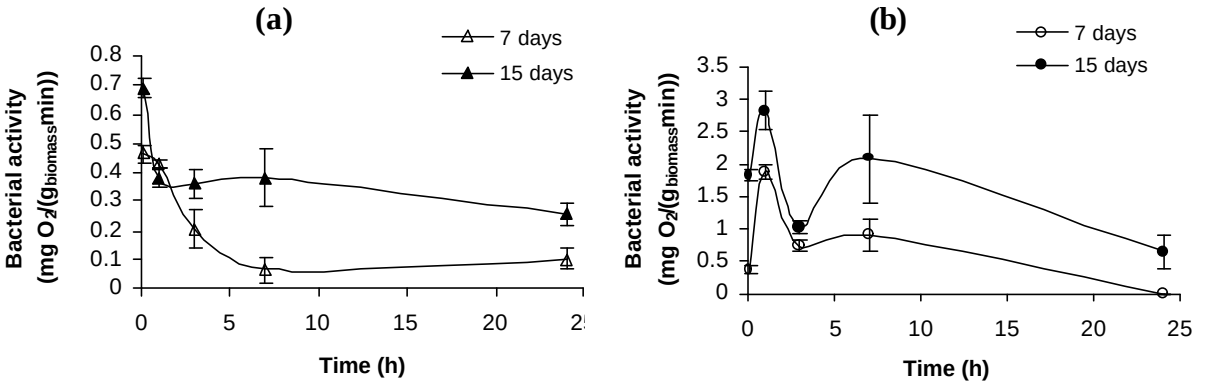

Figure 2. Bacterial activity values (bars represent the standard deviation) of the *P. fluorescens* biofilm when treated with 50 mg L^{-1} (a) (99% of confidence level for 4 degree of freedom) and 100 mg L^{-1} (b) of glutaraldehyde (95% of confidence level for 4 degree of freedom).

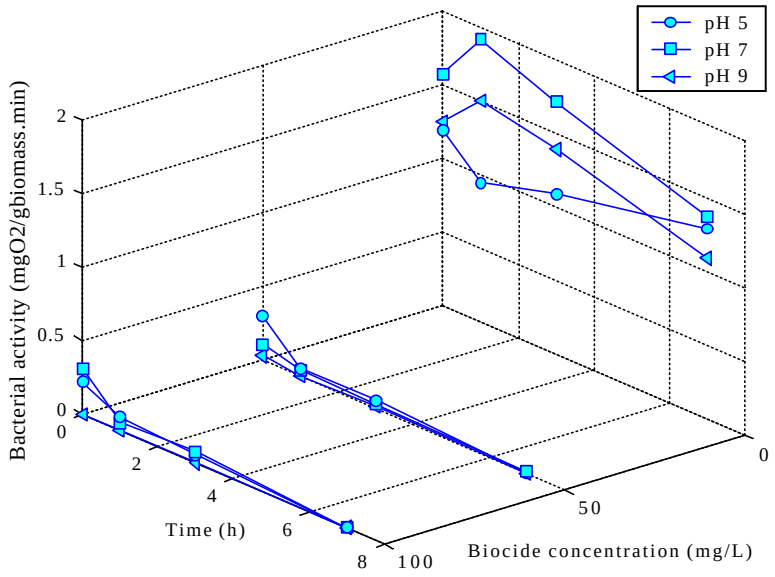

Figure 3. Bacterial activity of the suspended cultures of *P. fluorescens*, at several pH values, without biocide application and after treatment with 50 and 100 mg L^{-1} of glutaraldehyde, as a function of time.

Table 1. Characteristics of *P. fluorescens* biofilms. Mean values ± confidence interval of 95%.

Biofilm age	Biofilm mass	Biofilm activity	Protein ($mg_{protein}/g_{biofilm}$)			Polysaccharide ($mg_{polysaccharide}/g_{biofilm}$)		
(d)	($mg\ cm^{-2}$)	($mgO_2/(g_{biomass} min)$)	*Total*	*Intracellular*	*Extracellular*	*Total*	*Intracellular*	*Extracellular*
7	28.7 (± 8.4)	1.215 (± 0.488)	3.71 (± 0.72)	0.98 (± 0.61)	2.73 (± 0.91)	2.85 (± 0.81)	0.39 (± 0.89)	2.46 (± 1.53)
15	52.9 (± 11.6)	2.884 (± 1.002)	6.99 (± 2.77)	4.03 (± 1.52)	2.96 (± 0.54)	6.32 (± 1.98)	4.65 (± 0.83)	1.67 (± 1.04)

DISCUSSION AND CONCLUSIONS

These results suggest that, when GTA is used to control biofilms, it reacts not only with the proteins of the cells (the mechanism responsible for the lack of activity of the suspended bacterial cells treated with GTA) but also with the proteins of the polymeric matrix, leading to the disruption of the matrix structure, while bacteria remained attached to the surface. Thus, the GTA availability to bacteria decreases and consequently its antimicrobial action. These findings highlight that, besides the physical characteristics of the biofilms traditionally pointed out as responsible for the decrease in biocide efficacy, the biochemical composition of the matrix should also be taken into account in biocide treatment program (continuous application or intermittent dosing, the duration of the treatment, intervals between applications). Therefore, the efficacy of a chemical as a means to control biofilms should not be evaluated through the effect of the agent on the activity of planktonic cells. This approach may not reflect the action of the same chemical on biofilms formed by the microorganisms under study, since in biofilms there are parameters that might influence the action of the biocide.

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