

4^a Latin American Biodeterioration and Biodegradation
Symposium
Buenos Aires - AR, 16 al 20 de abril de 2001

APPLICATION OF MIXED TECHNIQUES TO ERADICATE *PSEUDOMONAS FLUORESCENS* BIOFILMS

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ABSTRACT

Early stages of *Pseudomonas fluorescens* (*P. fluorescens*) biofilms were studied using non-invasive techniques. The efficacy of glutaraldehyde (GA) against biofilms formed on stainless steel and on planktonic cells was analysed. Different biocide concentrations between (25 and 500 ppm) were assayed. As it was expected, the biocidal action was more effective in the case of planktonic cells and when higher concentration of the biocide was used in short periods. Results indicate that there was a definite biofilm resistance to GA and consequently highlights the need to optimise the application of this agent. To improve the biocidal efficacy against sessile bacteria mixed techniques (chemical-electrochemical) were employed. The killing action of biocide was enhanced when biofilmed samples immersed in a GA solution were exposed to an electric field.

INTRODUCTION

Biofilms accumulate in engineered systems such as heat exchangers, pipelines, processing equipments, storage tanks and induce technical problems including reduction in heat transference, increase in frictional resistance, corrosion and associated environmental damages and product contamination (McFeters et al., 2000; Geesey & Bryers, 2000). In medicine they are also responsible of chronic infections (Costerton et al., 1999; Werner et al., 1999; Costerton and Stewart, 2000).

The action of antimicrobial agents against sessile bacteria is universally found to be less effective than against planktonic cells. It seems that microorganisms resist the killing by biocides and antibiotics by a multiple resistance mechanism.

Glutaraldehyde (GA) is widely used as a biocidal agent in different environments like oilfields, industrial water treatments and sterilization of medical and odontological instruments (Grobe & Stewart, 2000). Considering that biofilms are not easy to eradicate by conventional GA treatments new techniques must be developed to achieve the complete killing of the cells. With this purpose, the application of a weak direct electric current previously employed to improve the efficacy of antibiotics (Stoodley et al., 1997; Wattanakaroon et al., 2000; Stewart et al., 1999) was assayed in this work to enhance the antimicrobial efficacy of GA. The early stages of *Pseudomonas fluorescens* (*P. fluorescens*)

biofilms were studied using non-invasive techniques. The efficacy of GA on biofilms formed on stainless steel and on planktonic cells was compared with that found when an electric field was applied.

EXPERIMENTAL

Pure cultures of *P. fluorescens* were used in the experiments. *P. fluorescens* was maintained in Cetrimide agar at 28°C and its purity was periodically checked by means of Gram stain, the oxidase test, and other biochemical tests and plating.

P. fluorescens inoculum was prepared by suspending a Cetrimide agar slant (24 h old) in 2 mL of sterile nutrient medium. The inoculum was later poured into an Erlenmeyer flask containing 200 mL of the nutrient broth medium and kept on a rotary shaker for 3 h.

The thin stainless steel coupons (area: 3 cm²) were immersed for 3 h in an Erlenmeyer flask inoculated with *P. fluorescens* under static conditions and biofilms formed on their surfaces. The number of planktonic bacteria at the beginning of the experiment was in the order of c.a. 10⁷ cells/mL. Occasionally 10⁹ cells/mL were assayed.

The biofilmed samples were treated in different ways:

Treatment I: The biofilmed samples were removed from the Erlenmeyer flask and afterwards they were immersed in 30 mL flasks containing a buffer solution (NaCl 8 g/L, KH₂PO₄ 0.34 g/L, K₂HPO₄ 1.21 g/L) with different GA concentrations (25, 50, 100, 200 and 500 ppm) and a control without GA. Planktonic cells that grew from the biofilmed coupons during GA treatment were enumerated after different periods (contact times: 1, 2, 4 and 6 h) using standard plating method for viable counts.

Treatment II: The biofilmed samples were put into an electrochemical cell containing a buffer solution and 100 ppm GA. A direct electric current was delivered to the chamber by means of a circuit containing a current controller and a platinum (anode) and a stainless steel (cathode) electrodes. Electric current flowed at a current density of c.a. 4 x 10⁻⁴ Acm⁻². The biofilmed samples were placed within the electrolyte volume confined between the electrodes.

Treatment III: After treatment I or II the biofilmed coupons were removed from the buffer and transferred to 30 mL flasks containing nutrient broth. The degree of growth of planktonic cells was considered as a degree of the reactivation capacity of the biofilm. Planktonic cells enumeration was made after 24 h and 48 h immersion periods in the nutrient broth.

The early stages of the biofilm growth were observed through a simple non-invasive optical microscopy method previously developed (Mele and Cortizo, 2000). The sample was

visualised as an opaque surface and the biofilm and the bathing solution as a bright area using a X40 objective.

RESULTS AND DISCUSSION

Different GA concentrations (25, 50, 100, 200 and 500 ppm) were assayed. In agreement with previous results (Grobe and Stewart, 2000) planktonic cells were readily killed by GA in this concentration range.

The killing action of GA against biofilms was indirectly analysed to avoid the scrapping of the samples which is sometimes unreliable (Costerton and Stewart, 2000). With this purpose, the biofilmed coupons were transferred to sterile buffer solutions with different GA concentrations (Treatment I) and the planktonic cells (coming from the biofilm) were enumerated. Fig. 1 shows that after 4 h of exposure planktonic cell number of the GA-containing buffer solution was at least 3 orders lower than that of the control.

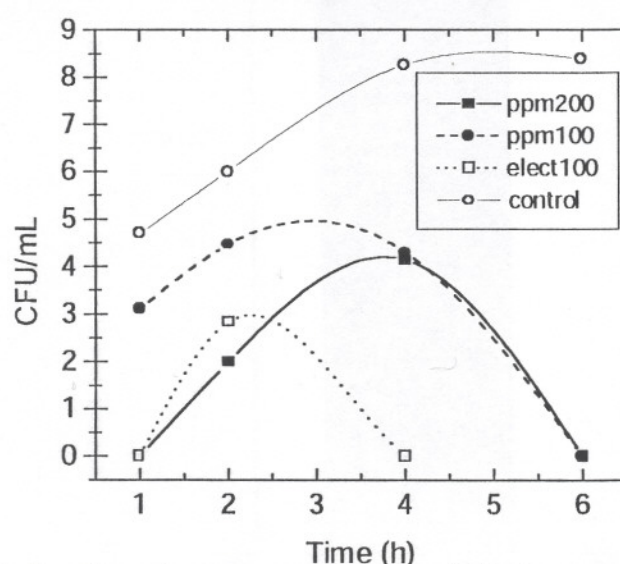


Fig. 1. Log₁₀ planktonic cell number vs. exposure time. a) Planktonic cells that grew from a biofilmed coupon after Treatment I with different GA concentrations: (○) control (0 ppm GA), (●) 100 ppm GA and (■) 200 ppm GA; and b) (□) Treatment II (100 ppm GA + electric field).

There were not planktonic cells alive after 6 h of exposure to 200 and 100 ppm when the initial planktonic cell number of the control was c.a. 10^7 CFU/mL. However, if this number was c.a. 10^9 CFU/mL (not shown), planktonic cells were not completely killed in the 100 ppm GA solution. The number of planktonic cells grown from the biofilm during the biocidal treatment seems to be related with the biocidal efficacy.

When the samples were immersed in a 100 ppm GA solution within an electric field (Treatment II) a significant reduction in the planktonic cells coming from the biofilm was observed (Fig. 1). Thus, the biocidal efficacy was enhanced by the electric field.

To accomplish the reactivation capacity of the biofilm after the immersion in the GA-containing buffer, Treatment III was applied. It could be noticed that planktonic cells grew in all cases even after the highest GA concentration, showing a recalcitrant resistance to GA treatment and a high reactivation capacity of the biofilm. Fig. 2 shows a dense hemispherical colony that was treated in a buffer solution with GA and then transferred to a nutrient broth. A slight layer of cells which seems to be floating in a viscous medium was found around the initial colony. They grew during the reactivation process of the biofilm in the nutrient broth. Although the penetration of the antimicrobial agent within the biofilm is reduced (Beyenal et al., 1998) it is not restricted enough to account for the highly reduced susceptibility of sessile microorganisms. Even the antimicrobial agent permeates readily, biofilm microorganisms

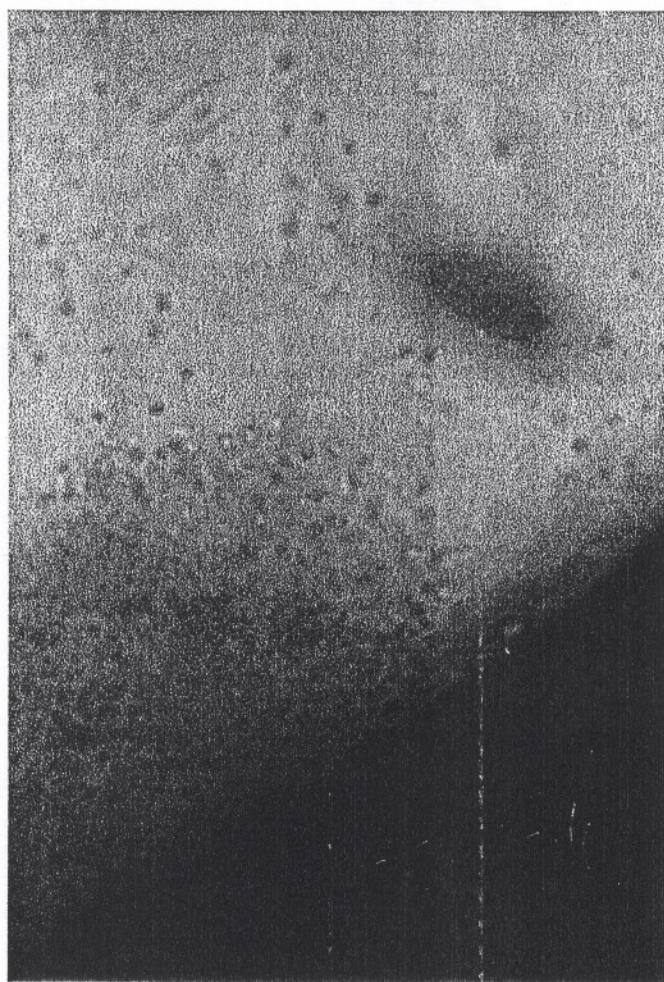


Fig. 2: *P. fluorescens* biofilm treated in a 25 ppm GA containing buffer solution (brown hemispherical colony at the left side) which was then transferred to a nutrient broth where a slight biofilm with lower cell density grew around it (brighter region at the top)

have shown recalcitrant resistance to killing (Viera et al., 1999). The major hypothesis that could explain the reduced susceptibility of the sessile cells shown in the present results

requires that some fractions of the cells adopt a slow-growing or starvation-like physiological state (Dodds et al., 2000). These cells may be the responsible of the biofilm reactivation after the GA treatment.

ACKNOWLEDGEMENTS

The authors wish to acknowledge CONICET, UNLP, CICPBA and to the National Agency for Promotion of Science and Technology (PICT 6782) for the financial support.

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