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Biocides effect on *Pseudomonas fluorescens* biofilms formed on glass surfaces

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Abstract

The objective of this study was to evaluate the resistance of Pseudomonas fluorescens biofilms to orto-phtaldehyde action under laboratory conditions, simulating a food processing environment. Biofilms were initially formed on glass coupons. Tests were performed using different concentration of biocide, biofilms of 6 day old and a range of exposure times. The data obtained suggested that the resistance of the treated biofilms to sanitizing agent may be due to attributes of extracellular polymeric substances. The results also demonstrated that the physical stability of the biofilms has been increased with biocide application and the biocide proved to be more effective for longer exposure times.

Keywords: biofilm, biocide, *Pseudomonas fluorescens*.

1. Introduction

Bacterial biofilms associated with surfaces are complex three-dimensional structures where bacteria are embedded in a matrix chiefly composed of extracellular polymeric substances (EPS) (Campanac et al., 2002).

Elimination of biofilm bacteria on processing equipment is a difficult task. Various methods such as mechanical treatment, as well as extra disinfection have been investigated in practice. The results show that a reduction in bacterial load could be achieved, but at present neither one single method nor one single chemical completely eliminated the microorganisms.

Attached microorganisms or microorganisms in biofilms can be a problem in food processing, because they adhere to the surfaces and if the cleaning is insufficient the remaining cells start to grow and contaminate the product (Hood et. all, 1995).

The general aims for microbial control including biofilm removal are to prevent spoilage of products and to ensure that the quality specifications of the product are met. The most important means for maintaining efficient microbial control include: minimizing the microbial load from outside sources to the process; efficient control of growth at microbiologically vulnerable sites and adequate cleaning and disinfection of the process lines (Wirtanen et al., 2000).

The effectiveness of cleaning was investigated through laboratory experiments using a generated biofilms. The effects of orto-phtaldehyde on a P. fluorescens biofilm model were studied.

2. Materials and Method

The type strain used was purchased from the American Type Culture Collection: *Pseudomonas fluorescens* ATCC 13525.

1. Orto-phtaldehyde action on P. fluorescens cells in suspension

Suspensions were prepared in sterile saline peptone solution (NaCl 8.5 g/L, peptone

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10.0 g/L), using fresh cultures grown on tryptic soy agar (TSA) medium at 36ºC for 24 hours.

One milliliter of the suspension obtained was transferred to test tubes containing 8 ml orto-phtaldehyde solution (10 mg/l, 25 mg/ml, 50 mg/ml and 100 mg/ml). The tubes were maintained at 25ºC in a thermostatic water bath. At every one minute, one tube was removed from the bath and the number of cfu/ml in each tube was determined by pour-plating the suspension on TSA medium and incubating at 35ºC for 48 hours. The experiments were repeated three times. Results correspond to average counts for each contact time between the sanitizer and the microorganisms.

2. Orto-phtaldehyde action on P. fluorescens biofilm

Biofilms were grown on glass coupons, with a surface area of 16 cm^2. Before each experiment every coupon was treated by immersion in acetone, air dried, rinsed with ultrapure water and finally immersed in HCl for 2 h, rinsed again with distillate water and air dried.

Overnight cultures of *Pseudomonas fluorescens* were grown in tryptone soya broth (TSB) at 35°C, using a shaker-incubator at 30 rpm. A 5 ml inoculum of the overnight culture was inoculated into two separate 1000 ml beakers each containing 500 ml TSB and vertically suspended glass coupons. The growth in the medium was monitored by absorbance at 580 nm and kept below 0.5 by addition of fresh medium. This was achieved by replacing the culture broth with the same volume of fresh medium every two days, for a total of six days.

After 6 day of growth, every test coupon were rinsed with distillate water, for planktonic cell removal and suspended in 30 ml of biocide (orto-phtaldehyde 100 mg/ml), for 2, 4, 6, 8, 10, 12, 14, 16, 22, 24, 30 and 35 min, followed by an immersion in neutralizing agent for 20 min.

Treated and control surfaces were each was repeatedly scraped by using a sterile spatula in order to recover attached cells in 20 ml distillate water for 1 min. Dislodged cells were enumerated, in duplicate, on TSA using a spread plating technique. Plates were incubated for 24-48 h at 35°C.

The efficiency of the process of biofilm scrapping and rapid evaluation of biocide activity on bacterial cells were ascertained by staining the glass coupon, after the process, with acridine orange (AO) using a microscope with phase contrast and epifluorescence Olympus BX 41. The data obtained was analyzed with TableCurve 2D, establishing linear regressions, that represent the destruction kinetics.

3. Results and Discussion

Cleaning and sanitizing may be the harshest stress that bacteria experience in a typical food processing environment. Therefore the objective of this study was to evaluate the resistance of *P. fluorescens* biofilms under laboratory conditions to a chemical agent.

1. Orto-phtaldehyde action on P. fluorescens cells in suspension

Figure 1 shows the efficiency of orto-phtaldehyde on *P. fluorescens* cells in suspension.

When *P. fluorescens* was treated with orto-phtaldehyde solution at 10 mg/l a reduction of 2.06 log cfu was attained in 10 minutes of contact. At 25 mg/l orto-phtaldehyde the reduction was 4.23 log cfu, which was attained in 10 minutes of contact.

An important reduction - 5.34 log cfu - was obtained at 50 mg/ml after 4 minute of biocide treatment. Total destruction of bacterial cells was observed at 100 mg/ml in 10 minutes of contact.

The graphics representations log cfu= f (τ) also show the destruction kinetics, which can be represented by first order equations. In Table 1 is represented the time necessary to obtain decimal reduction (D values).

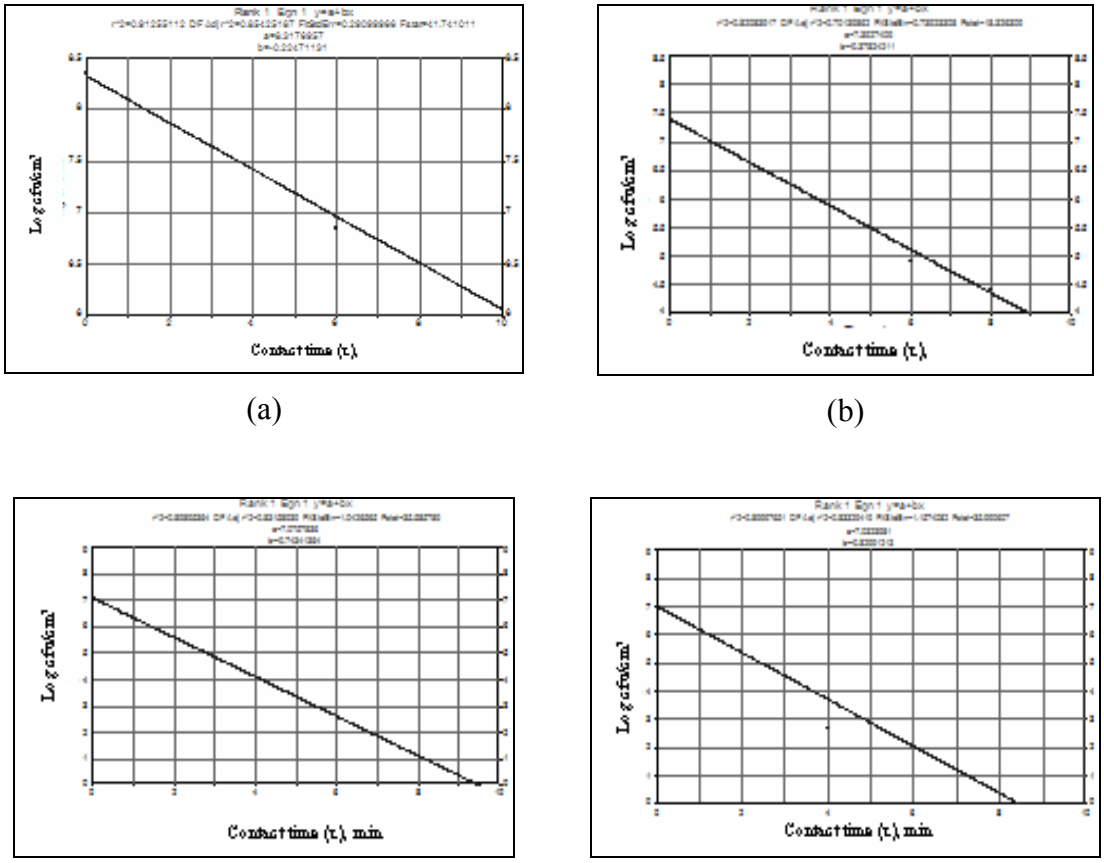

Figure 1 Action of orto-phtaldehyde 10 mg/l (a), 25 mg/ml (b), 50 mg/ml (c) and 100 mg/ml (d) on *P. fluorescens* in suspension at 25ºC.

Table 1. Destruction kinetics of *P. fluorescens* in suspension exposed to orto-phtaldehyde

Microorganism	Biocide concentration (mg/ml)	Linear regression	Regression coefficient (r^2)	D (min)
Pseudomonas fluorescens	10 mg/l	Log cfu/ml = 8.31 - 0.22·θ	0.912	4.85
	25 mg/ml	Log cfu/ml = 7.39 - 0.37·θ	0.820	2.36
	50 mg/ml	Log cfu/ml = 7.07 - 0.74·θ	0.898	1.26
	100 mg/ml	Log cfu/ml = 7.03 - 0.82·θ	0.899	1.19

θ - Contact time between the biocide and the microorganism

2. Orto-phtaldehyde action on P. fluorescens biofilm

The orto-phtaldehyde efficiency on *P. fluorescens* cells in suspension was different from that observed when the cells were forming biofilm on the glass surfaces (Figure 2). In this last condition, the destruction kinetic for the optimum concentration (100 mg/ml) established in previous method, it is represented by a different equation (Table 2).

An important reduction from 9.85 log cfu to 1.2 log cfu was obtained at 100 mg/ml concentration, after 35 minute of biocide treatment. The difficulty of removing *P. fluorescens* cells from glass using orto-phtaldehyde seemed to be associated with

changes to cell surface properties induced by the chemical agent used. According to Pasmore et al. (2002), the attraction between bacteria and the surface is expected also to play an important role in the ability to remove biofilms from a surface.

This increased resistance can be attributed to the protective barrier provided by the biofilm extracellular polymeric substances matrix.

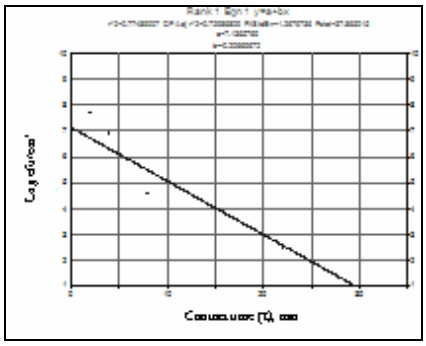

Figure 2 Action of orto-phtaldehyde (100 mg/ml) on *P. fluorescens* biofilm at 25ºC.

Table 2. Destruction kinetic of *P. fluorescens* biofilm exposed to orto-phtaldehyde

Microorganism	Biocide concentration (mg/ml)	Linear regression	Regression coefficient (r^2)	D (min)
Pseudomonas fluorescens	100 mg/ml	Log cfu/ml = 7.13 - 0.20·θ	0.774	4.04

θ - Contact time between the biocide and the microorganism

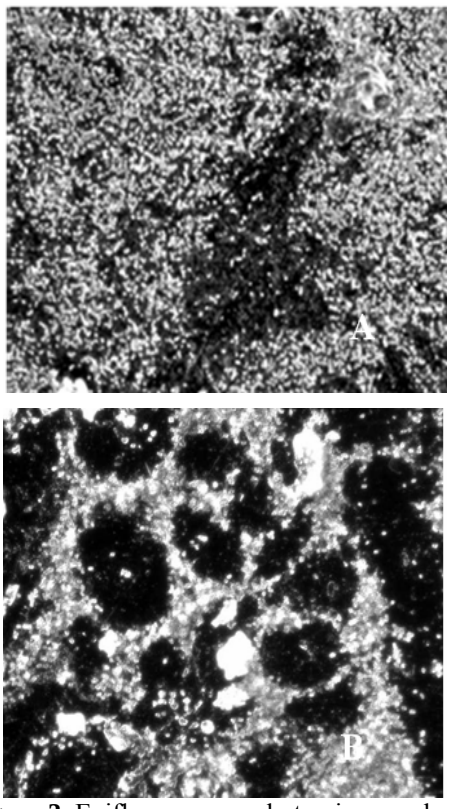

Figure 3. Epifluorescence photomicrograph of a 6 d old *P. fluorescens* biofilm formed on glass surfaces and stained with acridine orange before (A) and after (B) biocide treatment. X 100 magnification

Figure 3 shows representative microphotographs of biofilm formed on glass surfaces. The epifluorescence photomicrographs had been showed that after biocide treatment the biofilm matrix was not totally destroyed.

4. Conclusion

This study provides clear evidences of resistance to detachment of *P. fluorescens* attached to glass.

Although the antimicrobial chemical tested interacts strongly with bacterium, its application cannot be recommended for the induction of microbial detachment.

The results obtained for biofilm formed by *P. fluorescens* are a consequence of protective barrier provided by the extracellular polymeric substances matrix that hinders the action of the biocide.

This barrier is absent when the microorganisms are in suspension. Consequently, the destruction rate when the cells are in suspension is higher in this condition than when the microorganisms are forming biofilms.

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