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Effect of Biocides on Bacillus sp. Biofilms

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Abstract

The present study was to evaluate the bactericidal effects of biocides on Bacillus sp. biofilm formed on stainless steel. Tests were performed using different concentration of biocides, biofilms of 6 day old and a range of exposure times (from 0 (control) to 1, 5 and 20 minutes). The results provide clear evidences of resistance to detachment of Bacillus subtilis and Bacillus cereus attached to stainless steel, and for all the situations studied, bacterial biofilms were not completely removed (being still active) after biocide treatment.

Keywords: biofilm, biocide, *Bacillus*

1. Introduction

Bacteria form biofilms on the surfaces of stainless steel equipment in food processing industries, releasing bacteria that compromise the safety and quality of the final product [4]. Other unfavourable conditions associated with biofilms that affect food manufacturers include reduced flow through blocked tubes, reduced plant run times, corrosion of stainless steel, and reduced heat transfer through plate heat exchangers.

Biofilm bacteria live in a self-organized, cooperative community of microorganisms attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, and exhibit altered phenotypes with respect to growth rate and gene transcription [1,2].

Biofilm bacteria are notoriously tolerant to conventional chemical disinfectants [3]. These high tolerances may be caused by slow diffusion through the extracellular polymeric substance matrices, the existence of persisted cells, development of resistant phenotypes, and adaptations to micro-environments [5].

2. Materials and Method

The type strains used were purchased from the American Type Culture Collection: *Bacillus subtilis* ATCC 19659 and *Bacillus cereus* ATCC 10876.

Bacillus cereus was grown on sporulation media containing 10g/L beef extract plus 2g/L yeast extract and 15g/L agar at 37ºC for 7 days. Cells were harvested, washed three times and re-suspended in 5 ml distilled water. Suspensions were heated at 75-80ºC for 20 minutes and cooled immediately on ice for vegetative cells inactivation. Spores were stored as a dense suspension in distilled water, at 4ºC, until needed.

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

Overnight cultures of *Bacillus sp.* were grown in TSB at 35°C, using a shaker-incubator at 30 rpm. A 5 ml overnight culture was inoculated into two separate 1000 ml beakers each containing 500 ml TSB and vertically suspended stainless steel coupons.

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The growth in the medium was monitored by absorbance at 580 nm and kept below 0.5 by addition of a fresh medium. This was achieved by replacing the culture broth with the same volume of a fresh medium every two days, for a total of six days. The biofilms were allowed to grow for 6 days in order to obtain steady-state biofilms.

In the present study, for biofilm removal was used hydrogen peroxide (0.25%), sodium hypochlorite (2.5%) and a biocide combination (hydrogen peroxide 0.25% and sodium hypochlorite 2.5%). Every test coupon was rinsed with distillate water, for planktonic cell removal and suspended in 30 ml of biocide combination, followed by an immersion in neutralizing substances for 20 minutes. It was used as control 0.25% hydrogen peroxide and 2.5% sodium hypochlorite, for 1, 5 and 20 minutes.

The stainless steel coupons used were each was repeatedly scraped by using a sterile spatula in order to recover attached cells in 20 ml distillate water for 1 minute. Dislodged cells were enumerated, in duplicate, on TSA using a spread plating technique. Plates were incubated for 24-48 h at 35°C. The rapid evaluation of biocides activity on bacterial cells, was ascertained by coupons staining with 4`,6-diamidino-2-phenylindole (DAPI), using a microscope with phase contrast and epifluorescence Olympus BX 41.

3. Results and Discussion

Effectiveness of biocide combination, hydrogen peroxide and sodium hypochlorite against six days old *B. subtilis* biofilms on stainless steel coupons was tested at 1, 5 and 20 minute exposure times. The cellular characterization of 6 day old biofilms formed by *B. cereus* on stainless steel surfaces have been made by quantification of the colony forming unit, which was about 7.8 log CFU (Fig. 1).

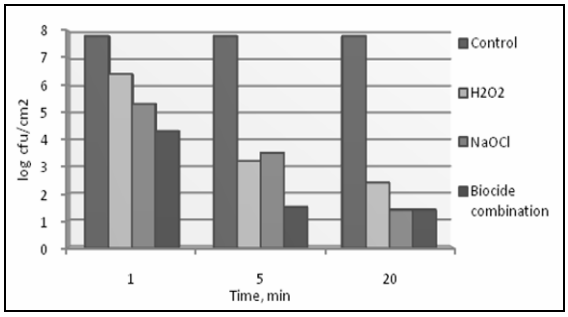

Figure 1. Biocides effect on biofilms formed by B. cereus on stainless steel surfaces

The effect of exposure time was also significant for stainless steel surface. Survival means of 6.4, 3.2 and 2.4 log CFU/cm^2 were observed for samples after 1, 5 and 20 minute contact time for hydrogen peroxide. However, a significant difference of the mean was only observed at 5 minute (3.5 log CFU) and 20 minute (1.4 log CFU) contact times for NaOCl.

The most effective sanitizer used against attached *Bacillus cereus* on surfaces tested, proved to be the biocide combination; obtaining after 5 minutes a 4.2 log CFU reduction and after 20 minutes a 6.5 log CFU reduction.

Biocide combination efficacy (Table 1) on attached bacteria was of ~45%, ~81% and 82%, after 1, 5 and respectively 20 minute contact time. In this case using the biocide combination on the bacterial population, had a significantly reducing of the cell number, comparing with disinfectants used individually.

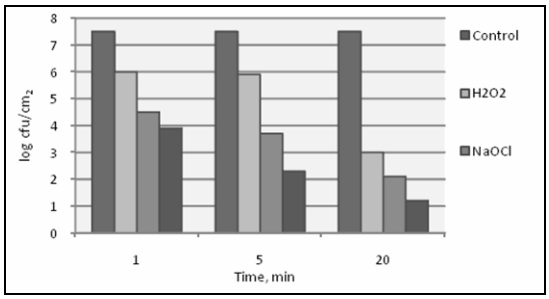

Figure 2. Biocides effect on biofilms formed by *B. subtilis* on stainless steel surfaces

The *Bacillus subtilis* biofilm attached cells were significantly reduced by using the biocide combination compared with hydrogen peroxide and sodium hypochlorite samples. A 5 and 20 minute contact time with the biocide combination reduced counts by 5.2 log CFU and 6.3 log CFU, respectively. However, for 20 minute contact time sodium hypochlorite and hydrogen peroxide only determined a 4.5 and 5.4 log CFU reductions, which were significantly different from the biocide combination.

Biocide combination efficacy (Table 1) on attached bacteria was of ~84% after 20 minute contact time. The biocide combination reduced significantly the bacterial population in biofilm comparing with disinfectants individually used.

In order to evaluate the biocide efficiency on bacterial cells, the biofilms were stained with DAPI and observed with epifluorescence microscopy, as shown in Figure 3 and Figure 4.

Table 1. Biocide combination efficacy 1:10 (0.25 % hydrogen peroxide: 2.5 % sodium hypochlorite) on bacterial biofilm

Biofilm	*B. cereus*, %			*B. subtilis*, %		
Contact time	H_2O_2	NaOCl	Biocide combination	H_2O_2	NaOCl	Biocide combination
1	17.9	32	44.8	20	40	48
5	58.9	55.1	80.7	21.3	50.6	69.3
20	69.2	82	82	60	72	84

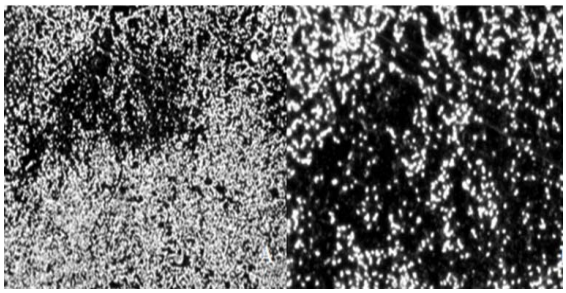

Figure 3. Epifluorescence photomicrograph of B. cereus biofilm before (A) and after the biocide combination action (B), contact time 20 minute

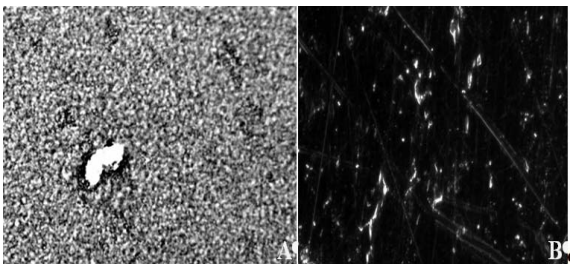

Figure 4. Epifluorescence photomicrograph of B. subtilis biofilm before (A) and after the biocide combination action (B), contact time 20 minute

On surface materials after treatment for 20 minutes with the biocide combination was observed a decrease in the number of stained cells.

4. Conclusion

Chemical antimicrobials are used to control pathogenic microbial populations in the environment. Peroxygens, such as hydrogen peroxide and hypochlorites, such as sodium hypochlorite are examples of chemical antimicrobials widely accepted as disinfectants in many industries.

Antimicrobial activity synergism between a mixture of hydrogen peroxide and sodium hypochlorite was confirmed against a wide variety of bacteria, both vegetative and spore forms.

Biocide combination reduced counts of *Bacillus sp.* biofilms on stainless steel surfaces. The biocide combination required to destroy tested biofilm bacterial was used in the ratio of 1:10 (hydrogen peroxide 0.25% and sodium hypochlorite 2.5%) necessary for removal of ~82% of *Bacillus cereus* biofilm and of ~84% of *Bacillus subtilis* biofilm, after 20 minute contact time.

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