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EFFECTIVENESS OF FREE CHLORINE AGAINST WATER BIOFILMS AND SPORES OF *PENICILLIUM BREVICOMPACTUM*

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INTRODUCTION

Biofilm is a microbial community characterized by attached cells on a surface or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they had produced and exhibit structural and phenotypic changes (Donlan and Costerton, 2002). Despite the fact that the term 'biofilm' is rarely applied to filamentous fungi, there are several descriptions indicative of biofilm formation in different medical, environmental and industrial settings (Mowat *et al.,* 2008a; Mowat *et al.,* 2008b). The occurrence of biofilms in water distribution systems can be a source of taste, odour and visual appearance problems resulting in poor drink water quality (Hageskal *et al.*, 2008). Moreover, fungi in potable water distribution systems may have direct effects on human health. Water for human consumption is often disinfected before goes to the distribution system. Chlorine, chloramines or chlorine dioxide are most often used because they are very effective disinfectants. In contrast, biofilms can protect microbes from disinfectants and allow microbes injured by environmental stress and disinfectants to recover and grow. The objective of this work was analyse the susceptibility of *Penicillium brevicompactum* biofilms and its single spores against free chlorine, the most common disinfectant used routinely in water treatment.

MATERIAL AND METHODS

Treatment With Free Chlorine

Spores: *Penicillium brevicompactum* (MUM 05.17) supplied by *Micoteca da Universidade do Minho* (MUM, Braga, Portugal) was chosen as a model as it is the most commonly filamentous fungi isolated from Portuguese tap water. Spores were collected from a 7-day pure culture at 25 ºC by adding 2 ml of distilled water to plate. The spore suspension was quantified using a Neubauer counter chamber. The suspensions were standardized by dilution with water to a final concentration of 10^5 spores/ml. Pellets of 10^5 spores were re-suspended in 1ml of free chlorine solution (Table 1), mixed by inversion to ensure full contact and then incubated at room temperature (25±1 ºC) for 15 min. Pellets were immediately washed with abundant distilled water and then centrifuged. This process was repeated three times. Finally the resulting pellets were re-suspended in 1 ml distilled water. Positive controls were treated in similar way, with the exception that distilled water replaced the free chlorine solutions.

Biofilms: The biofilms were grown in 6-well plates at room temperature and 120 rpm. The spore suspension was added to each well which contained 5 ml of glucose solution (0.1%). Then the PVC (polyvinyl chloride), PP (polypropylene) and PE (polyethylene) coupons (1 cm x 1 cm) were placed into the wells with the reverse face touching the well bottom and staying all under the water. After a time period 48h, 72h and 96h of incubation the biofilm on the coupons were used for free chlorine susceptibility test. The coupons were washed with distilled water to remove the non-adherent cells. Each coupon was transferred to another 6-well plate with different free chlorine solutions (0.02 mg/l, 1.57 mg/l and 2.38 mg/l) and submerged. After 15 min the coupons were taken off and washed three times with distilled water to remove the disinfectant. Both spores and biofilms were exposed to free chlorine solutions using the same conditions.

Concentrations of Free Chlorine

Sodium hypochlorite solutions were prepared with bleach and distilled and deionised water and adjusted to pH 7.0 ± 0.1 using HCl. The free chlorine concentration was measured with a colorimeter (Ion specific meters, Hanna Instruments, HI 93701, light emitting diode @ 555nm, range 0.00 to 2.5 mg/l, resolution 0.01 mg/L). A 10% sodium hypochlorite solution was used as negative control.

Viability Test

Culture test and FUN-1 staining (Molecular Probes, The Netherlands) were used to analyse the spores and biofilm viability.

RESULTS

Table 1 shows the sodium hypochlorite concentrations and the corresponding nominal concentrations of free chlorine. All disinfectant data refer to soluble free chlorine concentrations. The results after treatment with different free chlorine concentrations against biofilms and spores are shown in Table 2 and 3, respectively.

Table 1. Sodium hypochlorite and soluble free chlorine concentration used in the present study

SODIUM HYPOCHLORITE CONCENTRATION (% V/V)	FREE CHLORINE NOMINAL CONCENTRATION (mg/l)
1	2.38
0.5	2.13
0.25	1.98
0.125	1.83
0.6	0.25
0.3	0.05
0.015	0.02

Table 2. Survival of *P. brevicompactum* biofilms determined by germination capability after contact with different concentrations of free chlorine

	BIOFILMS								
	PVC			PE			PP		
Free chlorine (mg/l)	Biofilm age								
	48h	72h	96h	48h	72h	96h	48h	72h	96h
0,07	+	+	+	+	+	+	+	+	+
1,57	+	-	+	-	+	+	+	+	-
2,38	+	-	+	-	-	+	-	-	-
Positive control	+	+	+	+	+	+	+	+	+
Negative control	-	-	-	-	-	-	-	-	-

intensity of fluorescent signals was lower in the treated biofilms when compared with non treated biofilms. Nevertheless, the results obtained with treated biofilms after FUN-1 staining were conclusive under the conditions presented in this work. In conclusion, we presented a simple and reproducible methodology for the study of the effectiveness of free chlorine against filamentous fungi biofilms from water.

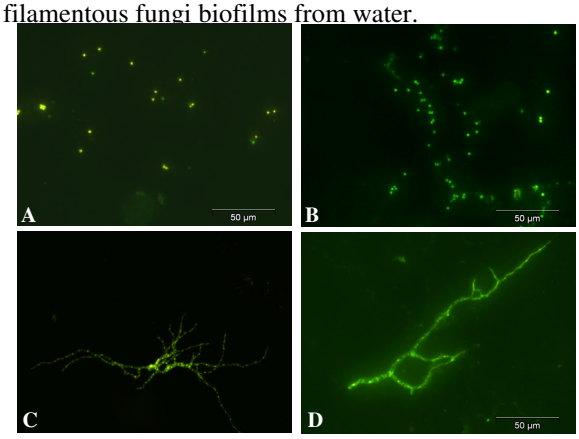

Figure 1. Spores exposed to 0.25 mg/l (A) and negative control (B); biofilm exposed to 1,83mg/l (C) and negative control (D) after FUN-1 staining.

For this, we applied conventional plating and FUN-1 staining and we have shown that FUN-1 is efficient and offered rapid and reliable results for laboratorial biofilms and more studies are necessary to apply the methods in real biofilms.

Table 3. *P. brevicompactum* spores enumeration after contact with different free chlorine concentrations

	ASSAY 1					ASSAY 2					ASSAY 3					
	REPLICATES			MEAN	STANDART DEVIATION	REPLICATES			MEAN	STANDART DEVIATION	REPLICATES			MEAN	STANDART DEVIATION	TOTAL MEAN (3 ASSAYS)
Free Chlorine mg/l	1	2	3			1	2	3			1	2	3			
2.38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.98	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.83	18	22	21	20,33	2,08	23	19	20	20,66	2,08	25	23	17	21,66	4,16	20,8
0.25	63	61	65	63	2	55	65	63	61	5,29	61	54	58	61	7	61,66
0.05	230	248	245	241	3,69	247	255	248	250	2,67	243	249	251	247,66	1,02	246,22
0.02	250	255	253	252,66	2,67	248	254	237	246,33	1,80	256	243	244	247,66	0,86	248,88
Positive control																2.5×10^2

After 30 min of incubation with FUN-1 viable spores from a 7-day pure culture were detected by conversion of FUN-1 dye into bright orange-red CIVS. The positive results allowed the comparison with treated spores. No evidence of autofluorescence was recovered in unstained spores. Effectiveness of free chlorine solutions against biofilms could be analysed by the FUN-1 staining under the conditions presented in this work (Figure 1). The results could be correlated qualitatively with conventional plating and was less time consuming.

Viable biofilms were detected after 30 min of incubation with FUN-1 by conversion of FUN-1 dye into bright orange-red CIVS. The results allowed the comparison with treated biofilms. We investigated whether FUN-1 can be used to detect viability after exposure to free chlorine solution. In general, the

Finally, *P. brevicompactum* biofilms were capable to survive after exposure to a high free chlorine concentration whereas free spores were susceptible.

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