WATER RESEARCH 43 (2009) 229–237

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Species association increases biofilm resistance to chemical and mechanical treatments

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ARTICLE INFO

Article history:
Received 30 July 2008
Received in revised form
3 October 2008
Accepted 7 October 2008
Published online 18 October 2008

Keywords:
Antimicrobial resistance
Biofilm control
Dual species biofilm
Hydrodynamic stress
Mechanical stability

ABSTRACT

The study of biofilm ecology and interactions might help to improve our understanding of their resistance mechanisms to control strategies. Concerns that the diversity of the biofilm communities can affect disinfection efficacy have led us to examine the effect of two antimicrobial agents on two important spoilage bacteria. Studies were conducted on single and dual species biofilms of *Bacillus cereus* and *Pseudomonas fluorescens*. Biofilms were formed on a stainless steel rotating device, in a bioreactor, at a constant Reynolds number of agitation (Re_A). Biofilm phenotypic characterization showed significant differences, mainly in the metabolic activity and both extracellular proteins and polysaccharides content. Cetyl trimethyl ammonium bromide (CTAB) and glutaraldehyde (GLUT) solutions in conjunction with increasing Re_A were used to treat biofilms in order to assess their ability to kill and remove biofilms. *B. cereus* and *P. fluorescens* biofilms were stratified in a layered structure with each layer having differential tolerance to chemical and mechanical stresses. Dual species biofilms and *P. fluorescens* single biofilms had both the highest resistance to removal when pre-treated with CTAB and GLUT, respectively. *B. cereus* biofilms were the most affected by hydrodynamic disturbance and the most susceptible to antimicrobials. Dual biofilms were more resistant to antimicrobials than each single species biofilm, with a significant proportion of the population remaining in a viable state after exposure to CTAB or GLUT. Moreover, the species association increased the proportion of viable cells of both bacteria, comparatively to the single species scenarios, enhancing each other's survival to antimicrobials and the biofilm shear stress stability.

1. Introduction

Bacillus cereus and *Pseudomonas fluorescens* are two major spoilage bacteria that produce tremendous process and end product quality problems in industrial systems (Dogan and Boor, 2003; Kreske et al., 2006). Their undesired effects are accentuated when they form biofilms. Once developed, their biofilms are harder to be eradicated and may serve as a chronic source of microbial contamination (Peng et al., 2002; Simões et al., 2005a). In fact, bacteria in biofilms have intrinsic mechanisms that protect them from even the most aggressive environmental conditions, namely the exposure to chemical antimicrobials (Gilbert et al., 2002; Cloete, 2003; Davies, 2003).

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doi:10.1016/j.watres.2008.10.010

Diversity in microbial communities leads to a variety of complex relationships involving inter and intraspecies interactions (Berry et al., 2006; Hansen et al., 2007; Elenter et al., 2007). The surface colonization by a bacterium can enhance the attachment of others to the same surface (Simões et al., 2007a). This process allows the development of multispecies communities often possessing greater combined stability and resilience than that of each individual species (Møller et al., 1998; Burmølle et al., 2006). There are some evidences that biofilm community diversity can affect subsequent disinfection efficacy (Leriche and Carpentier, 1995; Leriche et al., 2003; Burmølle et al., 2006). Nevertheless, the mechanisms regulating this phenomenon still remain unclear. Understanding single and multispecies biofilm survival in hostile environments should help the development of more efficient control strategies.

Chemical agents, such as biocides and surfactants, and mechanical forces are the main methods used to inactivate and remove biofilms (Cloete et al., 1998; Chen and Stewart, 2000; Simões et al., 2005a). Although the use of antimicrobial agents is widespread in biofilm control, standardized quantitative methods for antimicrobial selection and for the design of efficient biofilm control protocols do not exist. Consequently, strategies to remove unwanted biofilms must take into account the system characteristics (Stewart et al., 2000). It is expected that an effective and wide spectrum biofilm control strategy will overcome the problems of biotransfer potential (ability of any microorganisms associated with a surface that could eventually lead to the contamination of the processing product), cross-resistance and the existence of persistent biofilms (Verran, 2002; Gilbert and McBain, 2003; Simões et al., 2008a).

The objective of this study was to provide a better understanding of the effects of sequential antimicrobial and mechanical treatments on a dual species biofilm containing *B. cereus* and *P. fluorescens*. The characterization of single and dual species biofilms was performed to assess potential physiological aspects determining biofilm behavior to chemical and mechanical stresses.

2. Experimental procedures

2.1. Bacteria and culture conditions

P. fluorescens ATCC 13525^T and a *B. cereus* strain, isolated from a disinfectant solution and identified by 16S rRNA gene sequencing, were used throughout this study (Simões et al., 2007b). Bacterial growth conditions were 27 ± 2 °C and pH 7, with glucose as the main carbon source. Bacteria were grown in independent chemostats, consisting of 0.5 L glass chemostats (Quickfit, MAF4/41, England), with an air flow rate of 0.425 L/min and continuously fed with a sterile concentrated standard growth medium (glucose, 5 g/L, peptone, 2.5 g/L and yeast extract, 1.25 g/L, prepared in 0.02 M phosphate buffer, pH 7) (Simões et al., 2005b). The continuous feeding, with the aid of a peristaltic pump (Ismatec Reglo, Germany), occurred at a rate of 10 mL/h (*P. fluorescens*) or 13 mL/h (*B. cereus*) of sterile medium. Under the tested experimental conditions both bacteria had similar growth profiles and rates (Simões et al., 2008b).

2.2. Chemicals tested

The chemical agents used were the aliphatic cationic surfactant cetyl trimethyl ammonium bromide (CTAB; Merck, Portugal) and the aliphatic aldehyde-based biocide glutaraldehyde (GLUT; Reidel-de-Haën, Germany). Both chemicals were tested at 0.9 mM, obtained by preparation with sterile distilled water.

2.3. Single and dual species biofilm formation

Biofilms were grown on ASI 316 stainless steel cylinders, with a surface area of 34.6 cm^2 (2.2 cm diameter; 5 cm length), in a 3.5 L perspex (polymethyl methacrylate) bioreactor and rotating at a constant Reynolds number of agitation (Re_A; nondimensional parameter defined by the ratio of dynamic pressure and shearing stress) of 2400 (Fig. 1). This device offers a simple approach to study and characterize biofilms in a well-controlled, real-time and reproducible manner, and to mimic industrial flow processes (Azeredo and Oliveira, 2000). Three stainless steel cylinders were used in every experiment. Two biofilm containing cylinders were used for independent treatments with CTAB and GLUT and the other was used for biofilm phenotypic characterization. For single species biofilm formation, the 3.5 L bioreactor was continuously fed with sterile diluted medium (glucose, 50 mg/L, peptone, 25 mg/L, yeast extract, 12.5 mg/L, in 0.02 M phosphate buffer pH 7), and bacteria in the exponential phase of growth, supplied from the above-mentioned 0.5 L chemostats, at a flow rate of 10 mL/h for *P. fluorescens* or 13 mL/h for *B. cereus*, providing similar cell density inoculums. The flow rate of diluted medium was maintained at 1.7 L/h, so that it would support a cell density of 6×10^7 cells/mL for each bacterium. The biofilms were developed at 27 ± 2 °C, during 7 d in order to obtain biofilms in the phenotypic steady-state (Pereira et al., 2002).

For dual species biofilm formation, two independent 0.5 L chemostats were used to independently grow *B. cereus* and *P. fluorescens*. The 3.5 L bioreactor was inoculated simultaneously with the two bacteria, and fed with diluted nutrient medium at twice the flow rate (3.4 L/h) than the one used for single species biofilm formation, in order to obtain a cell density and residence time similar to that of the single species situation. The experiments were repeated at three different occasions for every scenario tested.

2.4. Biofilm sampling for phenotypic characterization

The biofilm (chemically untreated) on the stainless steel cylinders was removed using a stainless steel scraper and, afterwards resuspended in 10 mL of buffer solution (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7) and homogenised by vortexing (Heidolph, model Reax top) for 30 s with 100% power input, according to the methodology described by Simões et al. (2005a). The homogenised biofilm suspensions were then phenotypically characterized in terms of respiratory activity, total and extracellular polymeric substances (EPS) content (proteins and polysaccharides), and biomass amount and cell density. The *B. cereus* spore numbers in single and dual species biofilms were assessed by surface plating (300 μL sample) after biofilm suspension heat treatment (80 °C, 5 min). The plates of solid concentrated standard

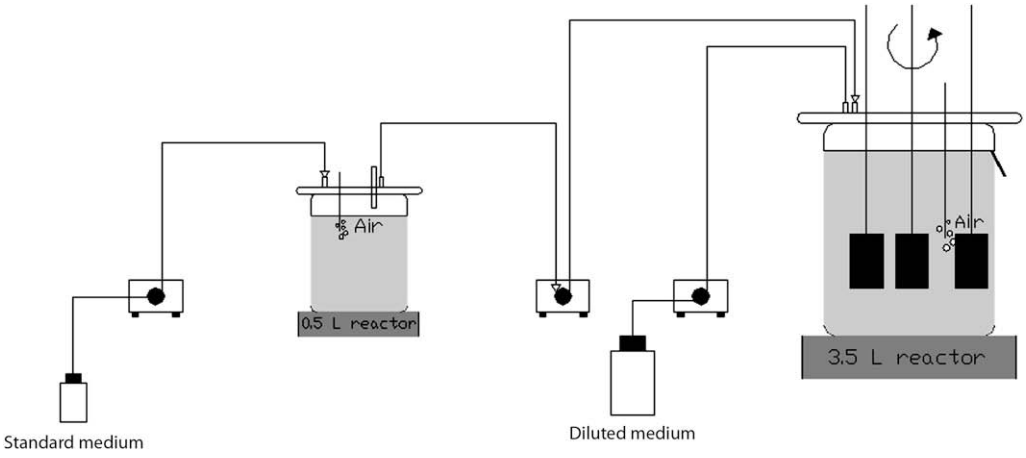

Fig. 1 – Schematic representation of the experimental system used to develop biofilms on the bioreactor rotating system.

growth medium (13 g/L agar) were incubated at 27 ± 2 °C for 72 h. The experiments were repeated at three different occasions by performing three independent biofilm formation experiments.

2.5. *Respiratory activity assessment*

Biofilm respiratory activity assays were performed in a model 53 Yellow Springs Instruments (Ohio, USA) biological oxygen monitor (BOM), as previously described (Simões et al., 2005c). Samples were placed in the temperature-controlled BOM vessel (27 °C ± 2 °C). Each vessel contained a dissolved oxygen (DO) probe, connected to a DO meter. Once inside the vessel, the samples were aerated for 30 min to ensure oxygen saturation ($[O_2]$ = 9.2 mg/L, 1 atm). Afterwards, the vessel was closed and the decrease of oxygen concentration monitored over time. The initial linear decrease observed corresponded to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, 50 μL of a glucose solution (100 mg/L) was injected into each vessel. The slope of the initial linear decrease in the DO concentration, after glucose addition, corresponded to the total respiration rate. The difference between the two respiration rates represented the oxygen uptake rate due to glucose oxidation and was expressed as $mgO_2/g_{biofilm}$ min.

2.6. *Proteins and polysaccharides quantification*

Biofilm EPS (proteins and polysaccharides) were extracted using Dowex resin (50 × 8, NA^+ form, 20–50 mesh, Fluka-Chemika, Switzerland), according to the methods of Frølund et al. (1996). Dowex resin was added to the biofilm suspensions. EPS extraction took place at 400 rpm and 4 °C for 4 h. The extracellular components (present in the supernatant) were separated from the cells via centrifugation (3777*g*, 5 min). The total (before EPS extraction) and extracellular biofilm proteins were determined using the Lowry modified method (Sigma, Portugal), with bovine serum albumin as standard. The procedure is essentially the Lowry method (Lowry et al., 1951) as modified by Peterson (1979). The total and extracellular polysaccharides were quantified through the phenol–sulphuric acid method of Dubois et al. (1956), with glucose as standard.

2.7. *Biomass quantification*

The dry mass of the biofilms was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions, according to standard methods (American Public Health Association [APHA], American Water Works Association [AWWA], Water Pollution Control Federation [WPCF]) (APHA/AWWA/WPCF, 1989). Following this methodology, the TVS assessed at 550 ± 5 °C in a furnace (Lenton thermal designs, UK) for 2 h is equivalent to the amount of biological mass. The biofilm mass accumulated was expressed in mg of biofilm per cm^2 of surface area of the slide ($mg_{biofilm}/cm^2$).

2.8. *Biofilm chemical treatment*

The cylinders with biofilm were removed from the 3.5 L bioreactor, and then immersed in 170 mL perspex vessels (diameter = 4.4 cm; length = 12 cm) containing CTAB or GLUT solutions. The biofilm exposure to chemical treatment was carried out with the cylinders rotating at a constant Re_A of 2400 for 30 min.

After biofilm chemical exposure, a neutralization step was performed to quench the chemicals antimicrobial activity, according to Johnston et al. (2002). CTAB was chemically neutralized by the following solution (w/v): 0.1% peptone, 0.5% Tween 80 and 0.07% lecithin (Sigma). GLUT was neutralized with sodium bisulphite (Sigma) at a final concentration of 0.5% (w/v).

2.9. *Biofilm removal by hydrodynamic stress*

Biofilm cells were removed by submitting the biofilms to increasing Re_A, according to the methodology described by Simões et al. (2005a). Following the chemical neutralization step, the cylinders with biofilm were inserted in the 170 mL vessels, now with 0.02 M phosphate buffer (pH 7) and consecutively subjected to a series of Re_A, i.e. 4000, 8100, 12,100 and 16,100, for a period of 30 s each. After each

hydrodynamic stress exposure, removed biofilm cells were collected by centrifugation (3777*g*, 5 min) and used to assess the number of total and viable cells, and the vessels were filled with fresh phosphate buffer. The residual biofilms, covering the cylinders, were entirely removed with a stainless steel scraper and resuspended into 5 mL of phosphate buffer, for cell enumeration and viability characterization. Three independent experiments were carried out for each biofilm type and chemical tested.

The amount of biofilm bacteria removed from the cylinder surface, after each Re_A, was expressed as the percentage of biofilm removal, and the biofilm bacteria that remained adhered to the cylinders after the serial hydrodynamic stress was expressed as the percentage of biofilm remaining according to the following equations:

$$\text{Biofilm removal}_i\,(\%) = (X_i)/(X_{\text{biofilm}}) \times 100 \quad (1)$$

$$\text{Biofilm remaining}\,(\%) = (X_{\text{remaining}})/(X_{\text{biofilm}}) \times 100 \quad (2)$$

X_{biofilm} – total biofilm cells; i – Re_A, i.e. 4000, 8100, 12,100 and 16,100; X_i – number of biofilm cells removed by a Re_A of 4000, 8100, 12,100 or 16,100; $X_{\text{remaining}}$ – number of biofilm cells remaining adhered to the stainless steel surface.

2.10. Enumeration of total and viable cells

Biofilm bacteria were stained with Live/Dead BacLight bacterial viability kit (Invitrogen/Molecular Probes, Leiden, The Netherlands), according to the procedure described by Simões et al. (2005c). This fast epifluorescence staining method was applied to estimate both viable and total counts of bacteria. *Bac*Light is composed of two nucleic acid-binding stains: SYTO 9™ and propidium iodide (PI). SYTO 9™ penetrates all bacterial membranes and stains the cells green, while propidium iodide only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells.

Biofilm samples were diluted to an adequate concentration (in order to have 30–250 cells per microscopic field), being thereafter microfiltered through a Nucleopore® (Whatman, Middlesex, UK) black polycarbonate membrane (pore size 0.22 μm), stained with 250 μL of SYTO 9™ solution and 250 μL of PI solution from the Live/Dead kit, and left in the dark for 15 min. A microscope (AXIOSKOP; Zeiss, Göttingen, Germany), fitted with fluorescence illumination and a 100× oil immersion fluorescence objective, was used to visualise the stained cells. The optical filter combination consisted of a 480–500 nm excitation filter, in combination with a 485 nm emission filter. Bacterial images were digitally recorded as micrographs using a microscope camera (AxioCam HRC; Zeiss). ScanPro 5 (Sigma) was used to quantify the number of cells and to measure the equivalent cell radius as an estimate of cell size (Walker et al., 2005).

B. cereus and *P. fluorescens* were distinguished according to the significant cell size differences (Simões et al., 2007b, 2008b). *B. cereus* biofilm cells had sizes of 1.58 ± 0.09 μm, while *P. fluorescens* had cell sizes of 0.583 ± 0.07 μm. The mean number of cells was determined from counts of a minimum of 20 microscopic fields. The proportion of viable cells in the removed/remaining biofilm layers was assessed as the ratio of viable and total cells for each bacterium in a specific layer.

2.11. Statistical analysis

The data were analysed using the Statistical Package for the Social Sciences, version 15.0 (SPSS, Inc, Chicago, IL). The mean and standard deviation within samples were calculated for all cases. The data were analyzed by the nonparametric Kruskal–Wallis test based on a confidence level ≥95%.

3. Results

3.1. Biofilms phenotypic characterization

B. cereus and *P. fluorescens* single and dual species biofilms were metabolically active oxidizing glucose as the main carbon source in the growth medium (Table 1). *P. fluorescens* biofilms were found to be five times more metabolically active resulting in higher biomass, cell density, and extracellular proteins and polysaccharides than *B. cereus* biofilms ($P < 0.05$). *P. fluorescens* biofilm matrix was highly composed of proteins (29% of the total proteins) and polysaccharides (61% of the total polysaccharides), while *B. cereus* biofilms had 8% of the total proteins and 10% of the polysaccharides as matrix constituents.

Dual species biofilms were about five times more metabolically active than *P. fluorescens* biofilms and had similar densities. The mass content was similar to those formed by *B. cereus* (Table 1). The dual species biofilm matrix had a significant proportion of both extracellular proteins (57% of the total proteins) and polysaccharides (53% of the total). Moreover, dual species biofilms were composed of log values of 13.9 (±0.1) and 13.6 (±0.09) cells/cm^2 of *B. cereus* and *P. fluorescens*, respectively. Spores were detected at numbers always smaller than 1×10^{-5}% of the vegetative *B. cereus* population in both single and dual species biofilms ($P < 0.05$).

3.2. Biofilm removal

The physical organization of the tested biofilms was found to occur in layers, showing different resistance to detachment by hydrodynamic stress (Fig. 2). Removal of *B. cereus* and *P. fluorescens* single species biofilms pre-treated with CTAB was higher for $Re_A \geq 12{,}100$, and dependent on the hydrodynamic stress increase ($P < 0.05$). Significant biofilm bacteria removal was achieved with the exposure to Re_A of 16,100 and 12,100, for *B. cereus* and *P. fluorescens* biofilms, respectively (Fig. 2a). Dual species biofilm removal, pre-treated with CTAB, was similar for the several Re_A.

Analysis of removal of GLUT pre-treated biofilms shows a higher removal of both single and dual species biofilms for the lower Re_A (Fig. 2b). Variability in the number of cells of each removed layer was found only for *B. cereus* biofilms ($P < 0.05$).

Biofilm removal results evidenced that their behavior face to shear stress changes, i.e. the biofilm mechanical stability, was higher for dual biofilms treated with CTAB (Table 2), with more than 66% of the total biofilm bacteria remaining adhered, and for *P. fluorescens* biofilms after GLUT exposure (more than 60% of the population remaining adhered). *P. fluorescens* and *B. cereus* single species biofilms held the lowest mechanical stability, after exposure to CTAB and GLUT, respectively.

Table 1 – Phenotypic characteristics of *B. cereus* and *P. fluorescens* single and dual species biofilms. The means ± SDs for at least three replicates are illustrated.

		B. cereus	*P. fluorescens*	Dual
Biofilm activity ($mgO_2/g_{biofilm}$ min)		0.0332 ± 0.0098	0.150 ± 0.022	0.813 ± 0.22
Log cellular density (cells/cm^2)		13.0 ± 0.21	14.0 ± 0.11	14.1 ± 0.091[a]
Biofilm mass (mg/cm^2)		0.413 ± 0.11	0.907 ± 0.093	0.506 ± 0.20
Proteins (mg/$g_{biofilm}$)	Total	205 ± 27	210 ± 19	321 ± 24
	Extracellular	15.8 ± 5.3	59.9 ± 15	184 ± 11.3
Polysaccharides (mg/$g_{biofilm}$)	Total	307 ± 33	200 ± 4.6	187 ± 17
	Extracellular	30.3 ± 4.2	121 ± 56	99.1 ± 16

a 13.9 of *B. cereus*; 13.6 of *P. fluorescens*.

3.3. *Biofilm viability*

B. cereus formed the most susceptibly biofilms to CTAB and GLUT, while dual species biofilms were the most resistant (Fig. 3). This phenomenon was observed invariably for the several biofilm layers (removed and remaining). The proportion of viable cells was significantly different when comparing the several layers ($P < 0.05$). A gradual increase in the proportion of viable cells was noticeable for the most inner layers. Furthermore, the proportion of viable cells was significantly different when biofilms were pre-treated with CTAB (Fig. 3a) or GLUT (Fig. 3b). In general, CTAB had a higher antimicrobial activity than GLUT ($P < 0.05$).

Viability of the remaining adhered biofilm layer (Table 2) reinforces the differential resistance/susceptibility between single species, and single and dual species biofilms. The remaining adhered layers of dual species biofilms treated with CTAB or GLUT had more than 95% of the total population in a viable state. This proportion of viable bacteria was significantly higher than those of single species biofilms ($P < 0.05$).

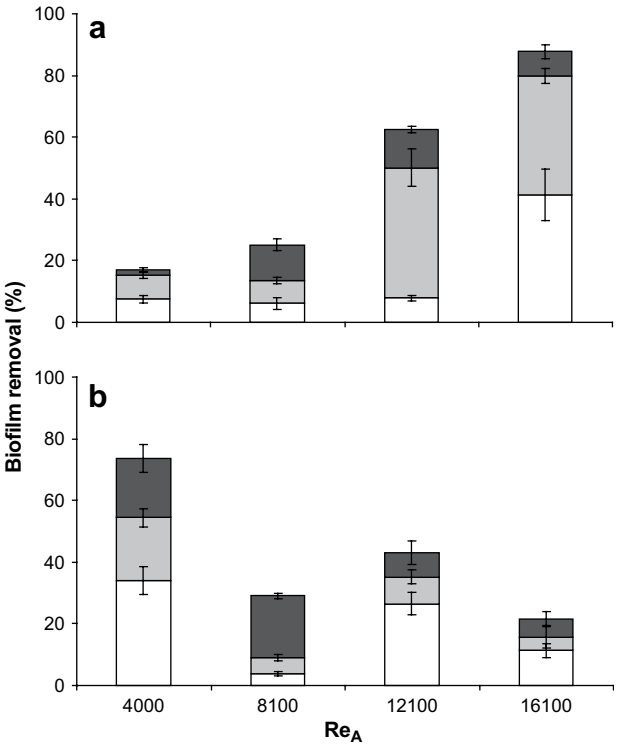

Fig. 2 – Biofilm removal after submitting the CTAB (a) and GLUT (b) treated biofilms to increasing Re_A. *B. cereus* (□) and *P. fluorescens* (▨) single and dual (■) species. The means ± SDs for at least three replicates are illustrated.

3.4. *B. cereus and P. fluorescens viability in dual species biofilms*

P. fluorescens cells in dual species biofilms outer layers were more tolerant to the tested antimicrobials than *B. cereus* (Fig. 4). A similar *B. cereus* and *P. fluorescens* tolerance to CTAB (Fig. 4a) was found in the inner layers removed by a Re_A of 12,100 and 16,100. The same effect was verified for GLUT exposed biofilms (Fig. 4b), and for the layers removed by Re_A of 8100, 12,100 and 16,100. Moreover, biofilm remaining were composed by a similar proportion of *B. cereus* and *P. fluorescens* viable cells (Table 2).

B. cereus and *P. fluorescens* in single (Fig. 3) and dual biofilms (Fig. 4), had distinct susceptibility to the tested chemicals. Both bacteria, in the several biofilm layers, were more resistant to the antimicrobials when in co-culture ($P < 0.05$). Comparisons between the proportion of viable bacteria in single and dual species biofilms evidence a protective effect of species association on bacteria viability after antimicrobial treatment.

4. Discussion

Control of microbial growth is required in many microbiologically sensitive environments, where wet or moist surfaces provide favourable conditions for microbial proliferation and biofilm formation (Verran, 2002; McBain et al., 2002). Biofilm control methods must take into account the knowledge of the constitutive microflora and their responsive behavior to control (Simões et al., 2005b, 2007a). In this work, the effect of shear forces' variation (through the increase in the Re_A) combined with the action of chemicals was investigated with *B. cereus* and *P. fluorescens* in single and dual species biofilms. The aim of the synergistic use of chemical treatment and mechanical action was to obtain bacteria-free surfaces.

The phenotypes displayed by single and dual species biofilms were significantly distinct. Dual species biofilms were primarily colonized by *B. cereus* and predominantly composed by EPS. The high biofilm cell counts reported are apparently

Table 2 – Percentage of remaining adhered (biofilm remaining) cells and respective viability of *B. cereus* and *P. fluorescens* from single and dual species biofilms exposed to the sequential CTAB/GLUT and mechanical treatments. Numbers which are in italics indicate the highest cell density and viability values.

	CTAB			GLUT		
	B. cereus	*P. fluorescens*	Dual	*B. cereus*	*P. fluorescens*	Dual
Cell density (%)	37.1 ± 3.3	4.21 ± 0.35	*66.3 ± 6.9*	24.1 ± 4.9	61.5 ± 1.8	47.2 ± 2.3
Viability (%)	70.5 ± 11	75.3 ± 6.5	*95.1 ± 4.3*[a]	77.7 ± 8.1	83.2 ± 7.8	*97.8 ± 1.3*[b]

a 94.1 ± 1.1% of *B. cereus* and 96.0 ± 3.3 of *P. fluorescens* biofilm cells in viable state.
b 97.3 ± 0.09% of *B. cereus* and 98.3 ± 1.3 of *P. fluorescens* biofilm cells in viable state.

related with the characteristics of the experimental system used. In fact, this bioreactor system and operating conditions were optimized to improve the potential of bacteria to form biofilms (Azeredo and Oliveira, 2000; Simões et al., 2005a, 2008b). While cell density differences of dual *B. cereus* and *P. fluorescens* biofilms are statistically significant, it seems unlikely that they are biologically and ecologically relevant. Dual species biofilms had a higher metabolic activity than single species biofilms, a phenomenon probably related with the distinct cell densities. Moreover, other phenotypic characteristics such as: increased biofilm porosity, growth kinetics and mass transfer efficiency could favour nutrient consumption and increase their metabolic activity (Melo and Vieira, 1999). The high EPS productivity of dual species biofilms, comparatively to the single species scenario, seems to be associated with the high metabolic activity. A previous study demonstrated the correlation between bacteria metabolic activity and EPS formation (Simões et al., 2007c). In fact, the metabolic activity is directly correlated with electron transport system activity (Babcock and Wikstrom, 1992). Other authors (Teo et al., 2000) demonstrated that proton translocation would induce the dehydration of cell surface, which could facilitate and strengthen the cell–cell interaction, and further lead to the creation of stronger and dense communities. However, other mechanisms could influence the differential EPS productivity in single and dual species biofilms. In fact, various specific pathways of biosynthesis and discrete export mechanisms involving the translocation of

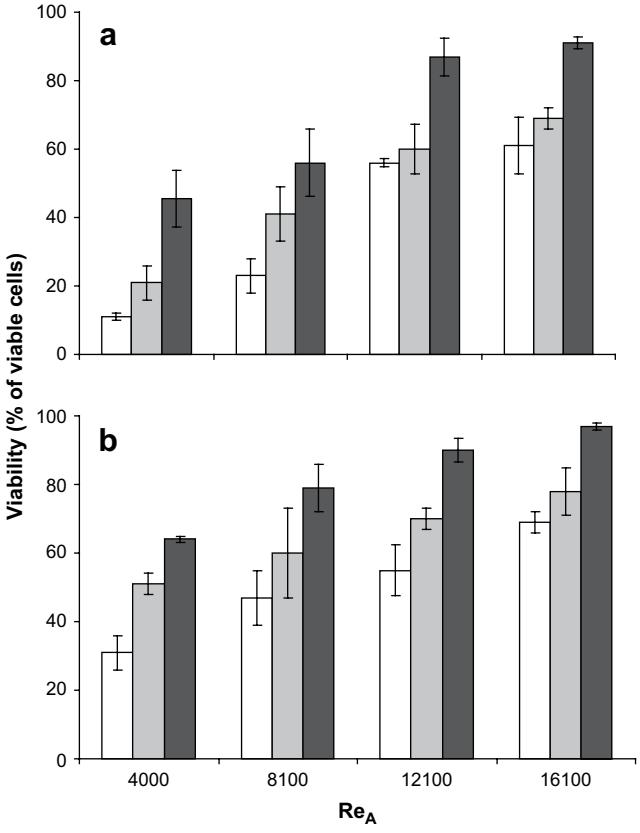

Fig. 3 – Viability (ratio of viable cells and total cells) of the biofilm layers removed by the series of Re_A after exposure to CTAB (a) and GLUT (b). *B. cereus* (□) and *P. fluorescens* (▒) single and dual (■) species biofilms. The means ± SDs for at least three replicates are illustrated.

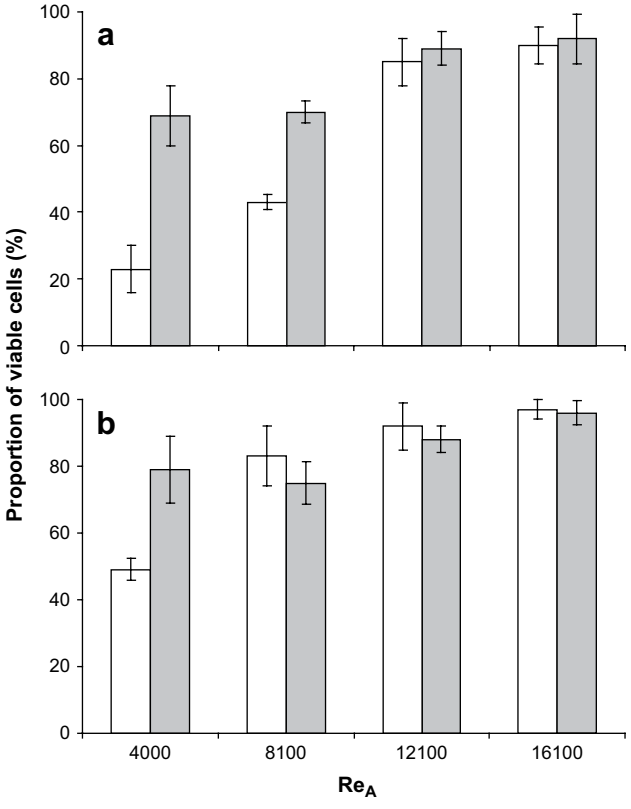

Fig. 4 – Proportion of viable *B. cereus* (□) and *P. fluorescens* (▒) cells in dual species biofilms exposed to CTAB (a) and GLUT (b). The means ± SDs for at least three replicates are illustrated.

EPS across bacterial membranes to the cell surface or into the surrounding medium have been described for bacterial proteins and polysaccharides (Beveridge et al., 1997; Osterreicher-Ravid et al., 2000; Nakhamchik et al., 2008). Some of the phenotypic characteristics studied, namely biofilm cell density, metabolic activity and EPS content, are relevant in biofilm control by conventional chemicals and by mechanical stress (Simões et al., 2005a,b). Moreover, spore formation was detected at low amounts in *B. cereus* single biofilms and in dual species biofilms. Ryu and Beuchat (2005) found similarly that in *B. cereus* biofilms, spores were at residual number comparatively to the vegetative cells.

The mechanical stability of biofilms was assessed by exposing them to different shear stresses in an attempt to weaken the biofilm structure and promote detachment. The biofilms layered structure had different susceptibilities to the sequential chemical and mechanical stresses. The resistance to removal was higher for dual species biofilms pre-treated with CTAB and *P. fluorescens* biofilms after GLUT exposure. *P. fluorescens* and *B. cereus* single species biofilms held the lowest mechanical stability, after exposure to CTAB and GLUT, respectively. According to some authors (Körstgens et al., 2001; Derlon et al., 2008), the removal of well-established biofilms requires overcoming the forces that maintain their integrity. Detachment of biofilms formed on the bioreactor rotating system is processed in layers, where the increase in the shear stress forces may progressively thin the biofilm (Azeredo and Oliveira, 2000; Simões et al., 2008b). This phenomenon is probably related with cylindrical geometry of the surfaces used for biofilm formation and with the massive detachment promoted by the shear stress forces. This detachment mechanism differs from that described for flowing systems, such as flow cells, where detachment of single cells and clusters are the main events (Stoodley et al., 2001). In the bioreactor rotating system, detachment of single cells and clusters were only significantly detected approximately 6 d after biofilm formation (time required to achieve the steady-state in terms of metabolic activity and cell density) and over time. However, those cells, removed by superficial erosion and by sloughing events, represented about $0.078 \pm 0.013\%$ of the total population. In fact, the amount of biofilm in a given system after a certain period of time depends on biofilm accumulation, which has been defined as the balance between bacterial attachment from the planktonic phase, bacterial growth within the biofilm and biofilm detachment from the surface (Stoodley et al., 1999). When that balance is null, the biofilm is said to have reached a steady-state (van der Kooij, 1999; Flemming, 2002).

In terms of viability, *P. fluorescens* was more resistant to antimicrobials than *B. cereus* in single species biofilms. Moreover, bacteria were more susceptible to antimicrobials in single biofilms than in the dual species biofilm system. Comparing surfactant and biocide antimicrobial action, CTAB was invariably more efficient than GLUT in biofilm bacteria inactivation. This is probably related with their distinct chemical classes and different antimicrobial mechanisms of action. GLUT has been a reference product for disinfection for many years, acting by cross-linking with functional proteins (Walsh et al., 1999; Fraud et al., 2001). CTAB is known to form electrostatic bonds and rupture cell membranes. The primary site of action of CTAB has been suggested to be the lipid components of the membrane causing cell lysis as a secondary effect (Gilbert et al., 2002; Simões et al., 2006). Both chemicals are known to interact strongly with proteins (Simões et al., 2006).

Bacterial tolerance to CTAB or GLUT was dependent on the cells location within the biofilm community. Biofilm inner layers were composed by a higher proportion of viable cells comparatively to the outer layers. In fact, bacteria in different zones of the biofilm pellicle experience different microenvironments and thus display different physiological behaviors (Stewart and Franklin, 2008). This physiological heterogeneity can be involved in the reduced antimicrobial susceptibility of bacteria in biofilms being the most reasonable explanation for the varying viability observed in the several biofilm layers. Nevertheless, the results also demonstrated that bacteria in dual species biofilms are even more resistant to killing and removal (only CTAB pre-treated biofilms) than single species biofilms. This increased resistance can be attributed to the protective barrier provided by the more abundant biofilm EPS matrix comparatively to single species biofilms. Also, the differential susceptibility of single and dual species biofilms to antimicrobials may be due to the EPS attributes, allowing a distinct interaction with the chemicals (Pan et al., 2006). In addition to the physical hindrance of antimicrobials diffusion caused by the EPS matrix, this barrier might also encompass others phenomena, such as absorption or catalytic destruction of the aggressor agent on the biofilm surface (Stewart et al., 2000). Moreover, the EPS plays a crucial role in maintaining the structural integrity of biofilms by both cellular adhesion and cohesion, allowing the formation of mechanically stable aggregates (Simões et al., 2005a). However, taking into account the biofilm phenotypic differences and the increased antimicrobial and mechanical resistance of dual species biofilms, comparatively to each single biofilm, it is possible that species association promotes other resistance mechanisms in addition to those promoted by the distinct described phenotypes. In fact, the complex biofilm architecture provides an opportunity for metabolic cooperation, and niches where antimicrobial-resistant phenotypes are formed within the spatially well-organized system (Davies, 2003; Klapper et al., 2007).

B. cereus and *P. fluorescens* in dual species biofilm inner layers had similar tolerance to both antimicrobials. This proposes that the predictability in CTAB and GLUT antimicrobial efficacy in multispecies aggregates is not only related with the bacterial cell composition and structure (*B. cereus* is Gram-positive while *P. fluorescens* is Gram-negative) and with the antimicrobial activity of the chemical agent, but with other events probably resulting from the species association. These interactions may lead to the formation of low susceptibility phenotypes. In fact, the results demonstrate that biofilm species association/diversity promotes community stability and functional resilience even after chemical and mechanical treatment. Similarly, Leriche and Carpentier (1995) demonstrated that *P. fluorescens* and *Salmonella typhimurium* in biofilm enhanced each other's survival following chlorine treatment. *Staphylococcus sciuri* was also found to protect *Kocuria* sp. microcolonies against a chlorinated alkaline solution (Leriche et al., 2003). Other apparent protective effects caused by bacteria association have been

mentioned (Lindsay et al., 2002; Whiteley et al., 2002). The synergistic species association found in this study, in addition to other well-described biofilm specific antimicrobial resistance mechanisms (Mah and O'Toole, 2001; Cloete, 2003; Davies, 2003; Klapper et al., 2007), could at least partly explain the survival of complex multispecies biofilms in adverse environments.

5. Conclusions

Single and dual *B. cereus* and *P. fluorescens* biofilms had significant phenotypic differences, mainly in the metabolic activity and both extracellular proteins and polysaccharides content. The physical organization of those biofilms was found to occur in layers showing different resistance to killing by CTAB and GLUT and detachment by hydrodynamic stress. *B. cereus* formed the most susceptibly biofilms to CTAB and GLUT, while dual species biofilms were the most resistant to antimicrobial action. In fact, dual species biofilms were more resistant to killing and physical removal (except GLUT pre-treated *P. fluorescens* single species biofilms) by shear forces than their respective single species biofilms. Moreover, the species association increased the proportion of viable cells of both bacteria, comparatively to the single species scenarios, enhancing each other's survival to the antimicrobials.

Acknowledgements

The authors acknowledge the financial support provided by the Portuguese Foundation for Science and Technology (Project Biomode-PTDC/BIO/73550/2006; PhD grant SFRH/BD/31661/2006 – Lúcia C. Simões).

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