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Effect of mechanical stress on biofilms challenged by different chemicals

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Abstract

In this study a methodology was applied in order to ascertain the mechanical stability of biofilms, by using a stainless-steel (SS) rotating device immersed in a biological reactor where biofilms formed by *Pseudomonas fluorescens* were allowed to grow for 7 days at a Reynolds number of agitation of 2400. The biofilms developed with this system were characterised in terms of amount of total, extracellular and intracellular proteins and polysaccharides, amount of mass, metabolic activity and mechanical stability, showing that the biofilms were active, had a high content of extracellular constituents and an inherent mechanical stability. In order to assess the role of chemical agents on the mechanical stability, the biofilms were exposed to chemical agents followed by mechanical treatments by submission to increase Reynolds number of agitation. Seven different chemical agents were tested (two non-oxidising biocides, three surfactants and two oxidising biocides) and their effects on the biofilm mechanical stability were evaluated. The increase in the Reynolds number increased the biofilm removal, but total biofilm removal was not found for all the conditions tested. For the experiment without chemical addition (only mechanical treatment), the biofilm remaining on the surface was about 76%. The chemical treatment followed by the subsequent mechanical treatment did not remove all the biofilms from the surface. The biofilm remaining on the SS cylinder ranged from 3% to 62%, depending on the chemical treatment, showing that the chemical treatment is far from being a cause that induces massive biofilm detachment and even the synergistic chemical and mechanical treatments did not promote biofilm removal. Some chemical agents promoted an increase in the biofilm mechanical stability such as glutaraldehyde (GTA), benzalkonium chloride (BC), except for the lower concentration tested, and sodium dodecyl sulphate (SDS), except for the higher concentration tested. Treatments that promoted biofilm removal, to an extent similar to the control experiment (without chemical treatment), were BC, for the lower and the higher concentration of SDS. Cetyltrimethyl ammonium bromide (CTAB), ortho-phthalaldehyde (OPA), sodium hydroxide (NaOH) and sodium hypochlorite (SHC) promoted the weakening of the biofilm mechanical stability.

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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). A better understanding of biofilm behaviour is particularly

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important due to the many serious problems associated with their presence (Simões et al., 2003b). The EPS matrix provides biofilm mechanical stability by filling and forming the space between the bacterial cells, keeping them together (Körstgens et al., 2001). Once developed, biofilms are harder to be removed completely (Simões et al., 2003b). Chemical agents and mechanical forces are parameters often involved simultaneously in the sanitation and removal of biofilms, since the application of sole chemical agents tends to leave the biofilm intact when no mechanical treatment is implemented in the control process (Flemming, 1996). Mechanical stability is an important factor in determining the structure and function of biofilm systems and this parameter plays a key role in the removal and/or control of biofilms in engineered systems (Poppele and Hozalski, 2003). So far, very limited studies have been conducted regarding the mechanical stability of biofilms (Körstgens et al., 2001; Ohashi and Harada, 1994, 1996; Ohashi et al., 1999; Poppele and Hozalski, 2003; Simões et al., 2003a, 2005b; Stoodley et al., 1999a). Moreover, studies concerning the effect of chemical agents on this biofilm parameter are even fewer. Physical forces acting on the biofilm can also influence the biofilm structure (Hall-Stoodley and Stoodley, 2002). One of the most important factors affecting biofilm structure and behaviour is the velocity field of the fluid in contact with the microbial layer (Pereira et al., 2002; Stoodley et al., 1999b; Vieira et al., 1993). The hydrodynamic conditions will determine the rate of transport of cells and nutrients to the surface, as well as the magnitude of shear forces acting on a developing biofilm.

In this paper, a reactor system that allows the formation and subsequent exposure of biofilms to different chemical and mechanical stresses is described. With this system, it is possible to assess the synergistic action of chemical and mechanical treatment on biofilm removal and to characterise the intrinsic biofilm mechanical stability.

2. Materials and methods

2.1. Microorganism and culture conditions

Pseudomonas fluorescens (ATCC 13525^T) was the microorganism used to produce biofilm. These bacteria are good biofilm producers and are one of the several microorganisms found in biofilms formed in industrial environments (Pereira et al., 2002). Their growth conditions were $27 \pm 1\,^{\circ}C$, pH 7, and glucose as the carbon source (Oliveira et al., 1994). The bacterial planktonic culture was grown in a chemostat, consisting in a 0.5 l glass reactor, continuously fed with a sterile concentrated nutrient solution—5 g/l glucose, 2.5 g/l peptone and 1.25 g/l yeast extract, in 0.02 M phosphate buffer (KH_2PO_4; Na_2HPO_4) at pH 7—at a flow rate of 10 ml/h.

2.2. Biofilm formation

Biofilms were grown on ASI 316 stainless-steel (SS) cylinders, with a surface area of 34.6 cm^2 (diameter = 2.2 cm; length = 5 cm), inserted in a 3.5 l reactor and rotating at 300 min^{-1}. Three SS cylinders were used in every experiment. This reactor was continuously fed (1.7 l/h) with sterile diluted medium, containing 50 mg/l glucose, 25 mg/l peptone, 12.5 mg/l yeast extract in phosphate buffer (pH 7, 0.02 M), and *P. fluorescens* in the exponential phase of growth supplied by the above referred 0.5 l chemostat at a flow rate of 10 ml/h. The biofilm was allowed to grow for 7 days before the assessment of the biofilm mechanical stability, in order to obtain steady-state biofilms (Pereira et al., 2001).

2.3. Mechanical stability of the biofilm

The mechanical stability of the biofilms was assessed by means of determining the biomass loss due to the exposure of biofilms to increasing Reynolds number of agitation in a rotating device described elsewhere (Azeredo and Oliveira, 2000). This device was already used to evaluate the mechanical stability of biofilms with and without chemical treatment (Simões et al., 2003b, 2005b). Biofilms were developed on three SS cylinders rotating at 300 min^{-1} and inserted in the above referred 3.5 l reactor (diameter = 16.8 cm). After 7 days of biofilm formation, the cylinders *plus* biofilm were carefully removed from the 3.5 l reactor. One of the cylinders was then immersed in a reactor with phosphate buffer (the control cylinder), while the others were immersed in reactors containing different chemical solutions (volume of each reactor was 170 ml). This chemical treatment was carried out with the cylinders rotating at 300 min^{-1} during 30 min. Afterwards, the cylinders were removed from the reactors containing the chemical solutions, accurately weighed, introduced in other reactors with phosphate buffer and consecutively subjected to serial velocities of rotation, i.e., 500, 1000, 1500, and 2000 min^{-1}, for a period of 30 s each. The wet weight of the cylinders *plus* biofilm attached was determined before and after each rotation. The experiments were repeated in three different occasions for every chemical treatment tested.

For each experiment, the SS cylinders were identified and weighed before being introduced in the reactor. The same procedure was followed with the control assay, i.e., with the cylinder *plus* biofilm immersed in the buffer solution.

The wet mass of the biofilm that was removed from the surface area of each cylinder, after each rotation speed, was expressed in percentage of biofilm removal, and the amount of biofilm that remained adhered after

Table 1
Reynolds number of agitation for each rotation speed used in this study

min^{-1}	$N'Re_A$
300	2400
500	4000
1000	8100
1500	12,100
2000	16,100

submission to the complete series of rotation speed was expressed as percentage of biofilm remaining, according to the following equations:

$$\text{Biofilm remaining (\%)} = (X_{2000} - X_c)/(X_{\text{after treat}} - X_c) \times 100, \quad (1)$$

$$\text{Biofilm removal}_{500\,\text{min}^{-1}}(\%) = (X_{\text{after treat}} - X_{500})/(X_{\text{after treat}} - X_c) \times 100, \quad (2)$$

$$\text{Biofilm removal}_{1000\,\text{min}^{-1}}(\%) = (X_{500} - X_{1000})/(X_{\text{after treat}} - X_c) \times 100, \quad (3)$$

$$\text{Biofilm removal}_{1500\,\text{min}^{-1}}(\%) = (X_{1000} - X_{1500})/(X_{\text{after treat}} - X_c) \times 100, \quad (4)$$

$$\text{Biofilm removal}_{2000\,\text{min}^{-1}}(\%) = (X_{1500} - X_{2000})/(X_{\text{after treat}} - X_c) \times 100, \quad (5)$$

where $X_{\text{after treat}}$ is the wet biofilm plus cylinder after the treatment during 30 min, X_c the wet masses of the cylinder, and X_{500}, X_{1000}, X_{1500}, X_{2000} are the wet masses of the biofilm plus cylinder after submission to, respectively, 500, 1000, 1500 and 2000 min^{-1}.

Assuming that the biological reactor had the behaviour of an agitated vessel, the Reynolds number of agitation ($N'Re_A$) as a consequence of each rotation speed can be calculated (Table 1) according to the following equation (Geankoplis, 1993):

$$N'Re_A = \frac{Da^2 N\rho}{\mu}, \quad (6)$$

where Da (m) is the diameter of the cylinder—$P>0.5$ when comparing $N'Re_A$ with and without the biofilm thickness (Pereira et al., 2002) associated with the diameter, N (s^{-1}) is the rotation speed, ρ (Kg/m^3) is the fluid density and μ (Kg/m s) is the fluid viscosity.

2.4. Chemicals tested

In the present work, the following chemical agents were used:

Two non-oxidising aldehyde-based biocides: Glutaraldehyde (GTA) that was purchased from Reidel-de-Haën (Cat. No. 62621) and the concentrations tested were 100, 200, 500 and 1000 mg/l.

Ortho-phthalaldehyde (OPA) that was purchased from Sigma (Cat. No. P-1378) and the concentrations tested were 50, 100, 200 and 300 mg/l.

Three surfactants: Cetyltrimethyl ammonium bromide (CTAB), a cationic surfactant, purchased from Merck (Critical micellar concentration—1.00 mM; Cat. No. 102342). The concentrations tested were 0.125, 0.250, 0.500 and 0.900 mM.

Benzalkonium chloride (BC), a cationic surfactant, purchased from Calbiochem (Critical micellar concentration—5.00 mM; Cat. No. 198901). The concentrations tested were 0.125, 0.250, 0.500 and 0.900 mM.

Sodium dodecyl sulphate (SDS), an anionic surfactant, purchased from Riedel-de-Haën (Critical micellar concentration—8.30 mM; Cat. No. 62862). The concentrations tested were 0.5, 1, 3 and 7 mM.

Two oxidising biocides: Sodium hydroxide (NaOH) purchased from Merck (Cat. No. 106467). The concentrations tested were 50, 200, 300 and 500 mM.

Sodium hypochlorite (SHC) purchased from Merck (13% active chlorine; Cat. No. 105614). The concentrations tested were 50, 200, 300 and 500 mg/l.

The concentrations of each product tested were obtained by preparation with sterile distilled water.

2.5. Biofilm characterisation

The biofilms that covered the SS slides were completely scraped from the metal slides, using a metal scrapper, resuspended into 10 ml phosphate buffer (pH 7, 0.02 M), homogenised in a vortex (Heidolph, model Reax top) for 30 s with 100% power input and used for further analysis. This biofilm suspension was used to assess the cellular respiratory activity of the biofilm through oxygen uptake rates and then biofilm mass. Biofilm from another cylinder was resuspended in extraction buffer for further quantification of its extracellular and intracellular proteins and polysaccharide content.

The experiments were repeated in three different occasions by performing three independent biofilm formation experiments.

2.6. Respiratory activity assessment

The respiratory activity of the biofilm was evaluated by measuring oxygen uptake rates due to glucose consumption in a biological oxygen monitor (BOM) in short-term assays. The assays were performed in Yellow Springs Instruments BOM (Model 53) and the procedure used is described elsewhere (Simões et al., 2003b). The biofilm samples were placed in the temperature-controlled vessel of the BOM ($T = 27\,°C \pm 1\,°C$). Each vessel contains a dissolved

oxygen (DO) probe connected to a DO meter. Once inside the vessel, the samples were aerated for 30 min to ensure the oxygen saturation. The vessel was closed and the decrease of the oxygen concentration was monitored over time. The initial linear decrease observed corresponds to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, a small volume (50 μl) of a glucose solution (100 mg/l) was injected within each vessel. The slope of the initial linear decrease in the DO concentration, after glucose injection, corresponds to the total respiration rate. The difference between the two respiration rates gives the oxygen uptake rate due to the glucose oxidation.

All the respirometric tests were carried out at least three times.

2.7. Extraction procedure

Extraction of the extracellular components of the biofilm was carried out using Dowex resin (50×8, NA^+ form, 20–50 mesh, Aldrich-Fluka 44445) according to the procedure described by Frølund et al. (1996). Prior to the extraction, the Dowex resin was washed with extraction buffer (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7). The biofilm was resuspended in 20 ml of extraction buffer and 50 g of Dowex resin per g of volatile solids were added to the biofilm suspension and the extraction took place at 400 min^{-1} for 4 h at 4 °C. The extracellular components were separated from the cells through centrifugation (3777*g*, 5 min).

2.8. Analytical methods

The chemical analyses were carried out on the homogenised biofilm suspensions. The proteins were determined using the Lowry modified method (SIGMA-Protein Kit no. P5656) and the polysaccharides by the phenol-sulphuric acid method of Dubois et al. (1956).

2.9. Biofilm mass quantification

The wet biofilm mass was assessed by the difference between the cylinder plus biofilm before the treatment and the clean cylinder.

The dry biofilm mass was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions, according to the Standard Methods (1989), method number 2490 A-D. The dry biofilm mass accumulated was expressed in g of TVS per cm^2 of surface area of the SS cylinder.

2.10. Statistical analysis

The data were analysed using the statistical program SPSS (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Because low sample numbers contributed to uneven variation, non-parametric Wilcoxon test procedure was used to compare the equivalence between the biofilm behaviour for the different rotation speeds for the same chemical concentration and for the same rotation speed for the different chemical concentrations. Statistical calculations were based on confidence level equal or higher than 95% ($P < 0.05$ was considered statistically significant).

3. Results

3.1. Characterisation of the biofilm formed on the rotating device

Fig. 1 shows a SS cylinder before the biofilm formation process (Fig. 1a) and a SS cylinder covered with biofilm after 7 days of growth (Fig. 1b).

This figure clearly shows that the surface of the SS cylinder was completely covered with a thick and slimy biofilm that seems to be strongly adhered to the surface. Some characteristics of the biofilms formed on the cylinders of the rotating device, namely the biofilm activity, mass, protein and polysaccharide content, are presented in Table 2. This characterisation was performed with biofilms before the submission to the chemical and mechanical treatments.

From Table 2 it can be verified that the biofilms were metabolically active, since it showed the ability to oxidise glucose (Simões et al., 2005a), and contained about 96% of water, which is in accordance with other authors (Vieira et al., 1993; Azeredo and Oliveira, 2000; Pereira et al., 2001). The amount of extracellular proteins was about 29% of the total biofilm proteins

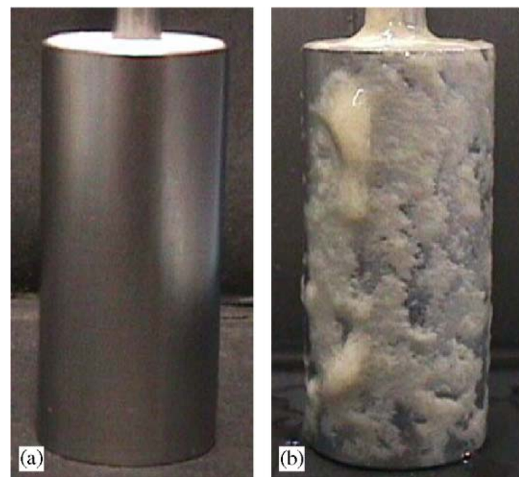

Fig. 1. Stainless-steel cylinder before the biofilm formation process (a) and covered with biofilm after 7 days of growth (b).

and the amount of extracellular polysaccharides was nearly 61.5% of the total biofilm polysaccharides. The total protein content was similar to the total polysaccharide content. However, since the analytical methods used to assess the amount of total proteins and polysaccharides were different, the comparison between quantitative amounts of proteins and polysaccharides cannot be accurately performed.

3.2. *Biofilm removal due to mechanical stress*

Fig. 2 shows the biofilm removal obtained due to the increase in the Reynolds number of agitation for the control experiment (without chemical treatment).

The existence of shear stress forces higher than the one under which the biofilm was formed ($N'Re_A = 2400$) caused biofilm removal. The high percentage of removal occurred with the implementation of a rotation of velocity that corresponds to a Reynolds number of 8100 (Fig. 2), being biofilm removal similar for the other Reynolds number tested. So, it can be said that the biofilm removal is dependent on the hydrodynamic conditions ($P<0.05$). Fig. 2 also showed that the total series of Reynolds number did not give rise to total biofilm removal, since only about 76% of biofilm mass was detached from the cylinders.

Table 2
Characteristics of the biofilm formed on the surface of the SS cylinders after 7 days of growth

Biofilm activity (mg O_2/$g_{biofilm\,min}$)	0.150±0.022	
Biofilm mass (mg/cm^2)	Dry	0.907±0.093
	Wet	21.5±6.1
Protein (mg/$g_{biofilm}$)	Total	210±19
	Extracellular	59.9±15
	Intracellular	150±17
Polysaccharides (mg/$g_{biofilm}$)	Total	200±4.6
	Extracellular	121±56
	Intracellular	79±18

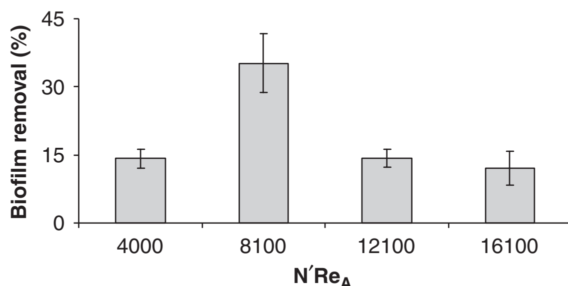

Fig. 2. Biofilm removal for the control assay due to change in the $N'Re_A$.

3.3. *Biofilm removal due to mechanical stress after exposure to non-oxidising biocides*

Figs. 3 and 4 show the biofilm removal caused by the implementation of the different Reynolds number after the biofilm was treated with GTA and OPA at different concentrations.

Fig. 3 shows that the total biofilm removal achieved with the total series of Reynolds number decreased with the increase of the GTA concentration used to previously treat biofilms. Moreover, for the lower $N'Re_A$ applied biofilm removal decreased with the increase in the GTA concentration. These facts suggest that the biofilm previously treated with GTA becomes less susceptible to the alteration of the shear forces. For each GTA concentration tested, the statistical analysis of the biofilm removal values achieved after each $N'Re_A$ showed that they were not equivalent ($P<0.05$). The comparison between the different GTA concentrations tested, for the same $N'Re_A$, shows that biofilm removal is significantly different ($P<0.05$). This result shows that GTA application and mechanical treatment had a significant effect on the biofilm removal.

The results obtained with OPA show that its application to biofilms favours the detachment caused by the change in the $N'Re_A$. The increase in this biocide concentration does not have a significant effect on the biofilm removal, since, for the same $N'Re_A$ (Fig. 4), the biofilm removal was similar for every concentration tested ($P>0.5$). It also can be noticed that the percentage of biofilm removal with the lower $N'Re_A$ applied increased when biofilms were previously treated with OPA. The comparison between the different $N'Re_A$ for the same OPA concentration shows that, only for 50 mg/l of OPA, the biofilm removal exhibited significant differences ($P<0.05$).

3.4. *Biofilm removal due to mechanical stress after exposure to surfactants*

Figs. 5–7 show biofilm removal caused by the exposure of the biofilm to the different Reynolds number after treatment with, respectively, CTAB, BC and SDS, at different concentrations.

CTAB enhances biofilm removal, with respect to the control that increases with CTAB concentration. The results also show that the biofilm removal takes place in a higher extent, for the smaller (4000 and 8100) $N'Re_A$ (Fig. 5). This trend becomes more important with the increase of CTAB concentration (Fig. 5). For the same CTAB concentration the biofilm removal values observed for the different $N'Re_A$ are statistically different ($P<0.01$), showing that CTAB application increases the

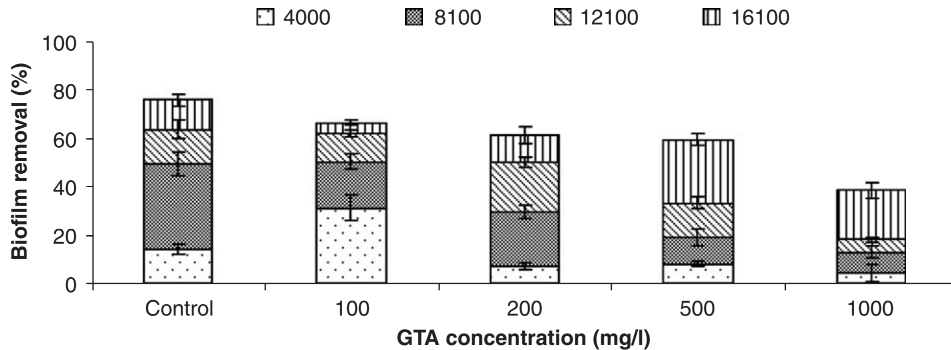

Fig. 3. Biofilm removal observed after the alteration of the $N'Re_A$ for the biofilm control and for the GTA treated biofilms.

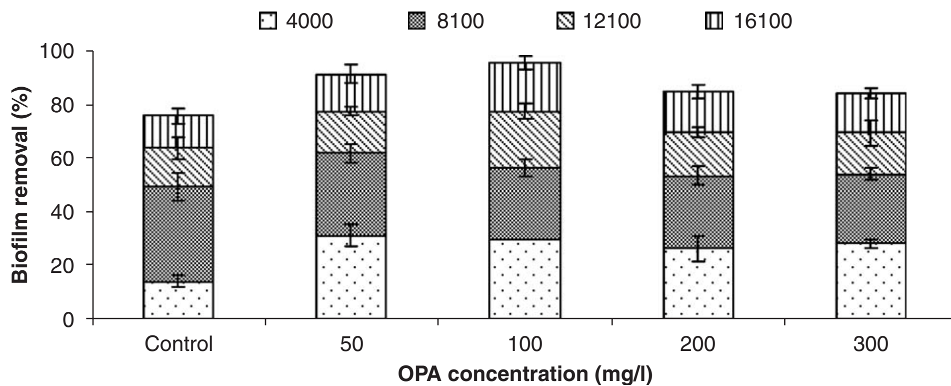

Fig. 4. Biofilm removal observed after the alteration of the $N'Re_A$ for the biofilm control and for the OPA treated biofilms.

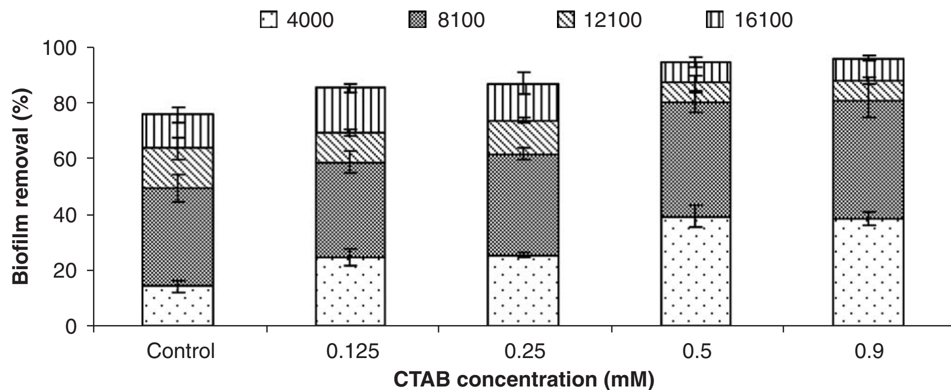

Fig. 5. Biofilm removal observed after the alteration of the $N'Re_A$ for the biofilm control and for the CTAB treated biofilms.

biofilm susceptibility to detachment through the mechanical action. However, when comparing the biofilm removal within concentrations and for the same $N'Re_A$, only for 4000 the biofilm removal was significantly different ($P<0.05$).

BC is a cationic surfactant as CTAB that caused different biofilm removal results. The increase in BC concentration used to treat biofilms increased the difficulty of biofilm removal through the alteration of the shear forces, especially when the lower $N'Re_A$ were implemented. Biofilm removal is equivalent ($P>0.10$) for the same $N'Re_A$ when comparing the different concentrations tested, except for 0.900 mM, where the differences are statistically significant ($P<0.05$). In this latter case, the higher amount of biofilm removal (30%) was found for the highest $N'Re_A$.

Concerning SDS, an anionic surface-active agent, apart from 7 mM, its application to the biofilm resulted

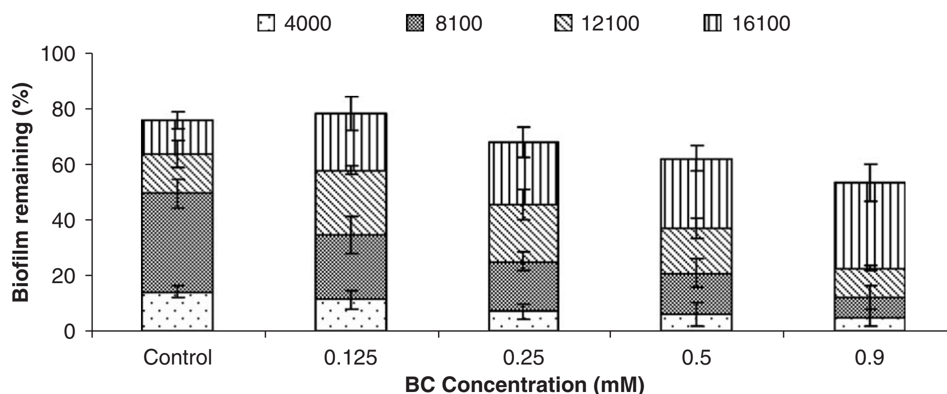

Fig. 6. Biofilm removal observed after the alteration of the $N'Re_A$ for the biofilm control and for the BC treated biofilms.

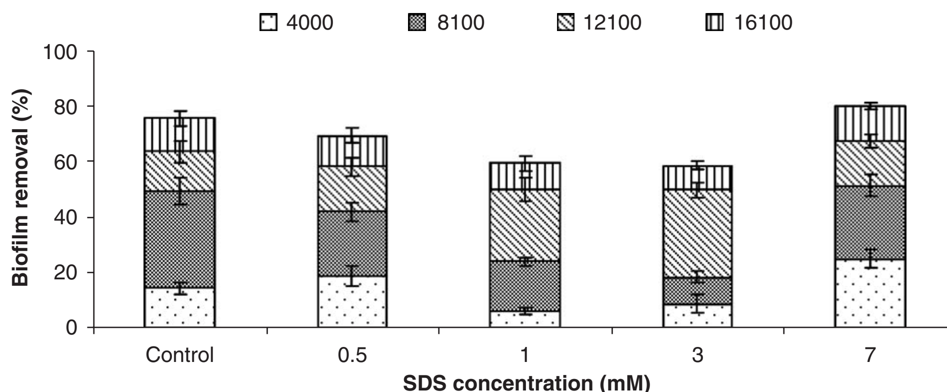

Fig. 7. Biofilm removal observed after the alteration of the $N'Re_A$ for the biofilm control and for the SDS treated biofilms.

in the decrease of biofilm removal achieved with the hydrodynamic change. Conversely, with the application of 7 mM of SDS biofilm removal takes place to a higher extent, for $N'Re_A$ of 4000 and 8100, but similar to the other $N'Re_A$ tested ($P>0.05$). For 0.500 mM the biofilm removal is similar for every $N'Re_A$ tested ($P>0.1$). The application of 1 and 3 mM of SDS promoted significant differences in the posterior biofilm removal ($P<0.05$), when comparing the different $N'Re_A$, being the high amount of biofilm removal promoted with the exposure to a $N'Re_A$ of 12,100. However, when comparing the biofilm removal for the same $N'Re_A$ within different concentrations, a significant difference ($P<0.05$) was found only for a $N'Re_A$ of 4000, due to the high amount of biofilm removal found after treatments with 0.5 and 7 mM.

3.5. *Biofilm removal due to mechanical stress after exposure to oxidising biocides*

Figs. 8 and 9 show biofilm removal caused by the exposure of the biofilm to the different $N'Re_A$ after treatment with NaOH and SHC at different concentrations.

Similar impacts on biofilm removal were found for NaOH and SHC (Figs. 8 and 9). Both chemicals similarly affected biofilm removal for every condition tested. Concerning NaOH, with the exception for 50 mM, the highest amount of biofilm removal is found for a $N'Re_A$ of 4000 and with the trend to increase with the increase in the concentration applied. For 50 mM the high amount of biofilm removal was found with an exposure to a $N'Re_A$ of 8100. However, the biofilm removal is statistically equivalent when compared with the other $N'Re_A$ ($P<0.05$). Concerning the comparison of the different $N'Re_A$ for the same NaOH concentration, the results are significantly different ($P<0.05$), with the exception for the treatment with 200 mM ($P>0.10$), where the biofilm removal happened to a similar extent with the submission to a $N'Re_A$ of 4000 and 8100.

The application of 50 mg/l of SHC resulted in a posterior biofilm removal that reached the highest amount with the exposure to a $N'Re_A$ of 8100. For the other concentrations tested, the biofilm removal was high for a $N'Re_A$ of 4000. The biofilm removal was

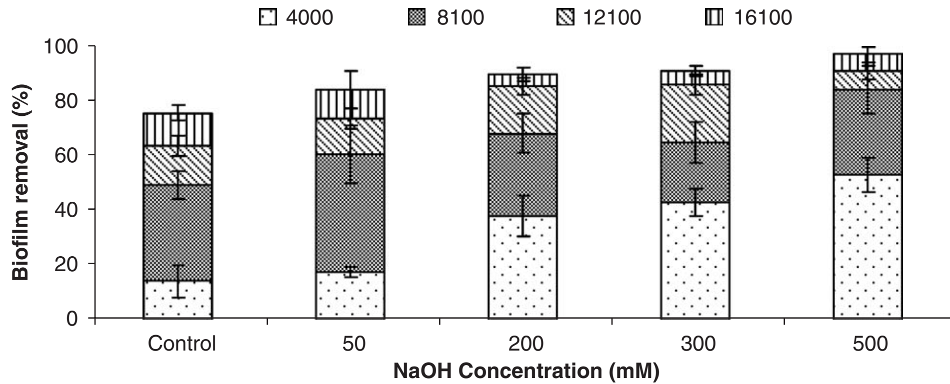

Fig. 8. Biofilm removal observed after the alteration of the $N'Re_A$ for the biofilm control and for the NaOH treated biofilms.

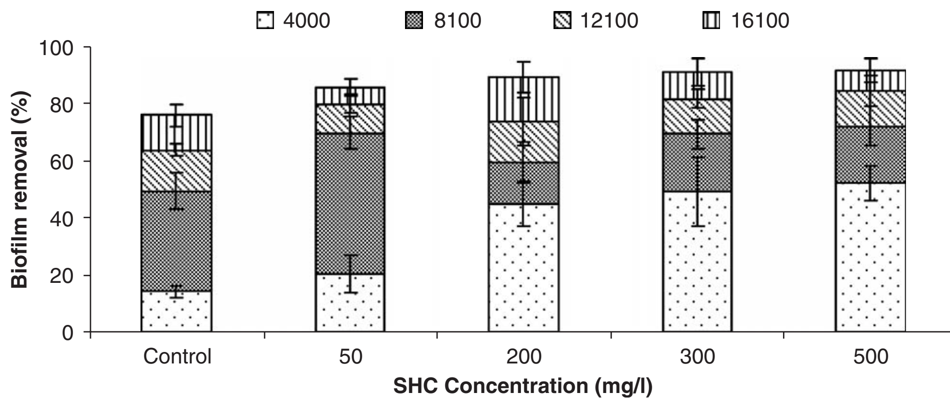

Fig. 9. Biofilm removal observed after the alteration of the $N'Re_A$ for the biofilm control and for the SHC treated biofilms.

similar for every concentration tested when comparing for the same $N'Re_A$ ($P<0.05$). The results were significantly different ($P<0.05$) as a consequence of the higher biofilm removal with the increase of $N'Re_A$.

3.6. Total biofilm remaining on the surface

The total percentage of biofilm that was not removed in the control experiment and for the experiments with the application of the different chemicals prior to the submission to the total series of $N'Re_A$, considered as the biofilm remaining, is presented in Table 3.

From this table, it is possible to emphasise that for the control assay, the biofilm remaining, after submission to the total series of $N'Re_A$ (Fig. 2) was about 24%. The addition of several chemicals to biofilms leads to different percentages of biofilm remaining that ranged from 3% to 62%. Treatments that promoted a similar or higher percentage of biofilm remaining than for the control assay were GTA for every condition tested, BC at 0.25, 0.5 and 0.9 mM and SDS at 0.5, 1 and 3 mM. The same range of values of percentage of biofilm remaining on the surface as the control assay were the experiments with 0.125 mM of BC and 7 mM of SDS.

4. Discussion

The characteristics of the biofilms formed on the SS cylinders (Table 2), namely the respiratory activity, biofilm mass and total content of proteins and polysaccharides, are similar to the ones observed in biofilms formed in a flow cell system under turbulent flow (Simões et al., 2003a), specifically the significant content of extracellular proteins and polysaccharides found in the composition of the biofilm matrix. The evidence of the slimy matrix of the biofilm depicted in Fig. 1 acquired great importance in biofilm architecture; thus, in biofilm mechanical stability, since, according to Körstgens et al. (2001), EPS are responsible for keeping biofilm together and binding the biofilm to the support, forming a temporary network of fluctuating junction points. The mechanical stability of biofilms, i.e., the behaviour of biofilms facing external stress mechanical conditions, is of great impact for both wanted and

Table 3
Total percentage of biofilm remaining on the surface for the several chemical treatments and for the control experiment after the submission to the total series of $N'Re_A$

	Treatment	Total biofilm remaining (%)
	Control (without chemical treatment)	24.2±0.59
Non-oxidising biocide	*GTA*	
	100 mg/l	33.8±3.3
	200 mg/l	35.4±9.9
	500 mg/l	40.6±2.1
	1000 mg/l	61.5±1.8
Non-oxidising biocide	*OPA*	
	50 mg/l	15.6±4.3
	100 mg/l	14.3±3.3
	200 mg/l	14.8±5.6
	300 mg/l	15.0±3.1
Cationic surfactant	*CTAB*	
	0.125 mM	14.8±1.3
	0.250 mM	13.2±2.8
	0.500 mM	5.31±0.72
	0.900 mM	4.16±0.35
Cationic surfactant	*BC*	
	0.125 mM	21.8±6.5
	0.250 mM	32.0±7.9
	0.500 mM	37.9±8.7
	0.900 mM	46.7±12
Anionic surfactant	*SDS*	
	0.500 mM	30.6±4.5
	1.00 mM	40.7±4.2
	3.00 mM	41.6±6.2
	7.00 mM	19.7±3.9
Oxidising biocide	*NaOH*	
	50 mM	15.7±4.8
	200 mM	10.1±3.9
	300 mM	8.63±1.7
	500 mM	2.89±2.1
Oxidising biocide	*SHC*	
	50 mg/l	14.1±5.4
	200 mg/l	10.8±4.9
	300 mg/l	8.95±5.6
	500 mg/l	8.48±1.9

unwanted biofilms (Poppele and Hozalski, 2003). In this study, the mechanical stability of the biofilm was assessed by submitting biofilms to different shear stresses, corresponding to increasing $N'Re_A$, which may weaken the biofilm structure and promote detachment. The biofilm formed on the cylinders of the rotating device prior to chemical stress was characterised in order to determine the inherent biofilm mechanical stability, since detachment processes may be dependent on it. According to Stoodley et al. (1999a), biofilm matrix develops an inherent internal tension, which is in equilibrium with the shear stress under which the biofilm is formed. The EPS strengthen the cohesive forces within the biofilm, thereby contributing to an enhanced inherent biofilm mechanical stability (Azeredo and Oliveira, 2000). The removal of a well-established biofilm requires to overcome the forces which maintain the integrity of the biofilm (Körstgens et al., 2001). The control experiment (Fig. 2) showed that biofilms subjected to sole mechanical treatment were hardly removed with low shear stress ($N'Re_A \leqslant 4000$) since only about 14% of biofilm removal was achieved. However, when the $N'Re_A$ were raised from 4000 to 8100 a noticeable biofilm detachment was observed, but a layer remained on the surface even when the highest $N'Re_A$ was applied. According to Azeredo and Oliveira (2000), the biofilm detachment is processed in layers, where the increase in the shear stress may progressively thin the biofilm, mechanical failure and total detachment being the ultimate effects expected. The removal of biofilms from surfaces using increasing shear stress promoted by the increasing in the $N'Re_A$ is a mechanical phenomenon. However, the most common practice to eliminate unwanted biofilms involves the application of toxic chemicals (Chen and Stewart, 2000). Previous studies (Simões et al., 2003a, b, 2005b) showed that chemical agents display only limited ability to remove biofilm layers, even though biofilms may be inactivated. After the chemical treatment of the biofilm, their EPS matrix often remains more or less unaffected and thus biofilm is left in place (the amount of biofilm removed due to the exposure to the chemical agents during 30 min was about 5±2% for every condition tested). This biofilm can act as an additional source of nutrients and/or as a suitable surface to further growth of cells. The regrowth of the injured microorganisms (Simões et al., 2005b) can also be stimulated in these biofilms. So, in this work, together with shear forces variation (through the increase in the $N'Re_A$) the coupled action of a set of chemicals in biofilm stability was also investigated. The desired ending of the synergistic use of chemical treatment and mechanical action was to have a clean surface. Besides the chemical agents could interact with the cohesive forces of the biofilm, causing the destabilisation of the structure, the synergistic action of chemical and mechanical treatment was the main strategy for biofilm control.

Concerning aldehyde compounds, OPA is used as a possible alternative to GTA for high-level disinfection (Simons et al., 2000; Walsh et al., 1999a, b). The bi-functional nature of GTA allows it to react and cross-link with ammonia and primary amine groups and more slowly with secondary amines (Walsh et al., 1999b; McDonnel and Russell, 1999). Following the hypothesis of GTA as a cross-linking agent would lead to predict

that biofilm treatment with GTA should actually stabilise the biofilm, as found with this work. GTA was not efficient in removing the biofilm from the SS cylinders in spite of the fact that this biocide is frequently used to chemically control the accumulation of biofilms (Pereira and Vieira, 2001). On the contrary, GTA contributed to the formation of a harder deposit, since the percentage of biofilm remaining on the surface was higher than for the control experiment.

Conversely, the results obtained with OPA are consistent with its less effect of cross-linking when compared with GTA. Probably this fact is related with the aromatic ring presented in the molecular structure of OPA, which confers a diminished flexibility of the molecule, conversely to the aliphatic chain of GTA (Simons et al., 2000; Walsh et al., 1999a, b). Consequently, the biofilm remaining on the surface decreases slightly after OPA application in relation to the control.

The treatment with surfactants caused different biofilm responses that may be related with their chemical nature. Concerning the cationic surfactants, the behaviour of CTAB differs significantly from the one observed with BC. The action of those cationic surfactants is attributed to their positive charge that forms an electrostatic bond with negatively charged sites (Cloete et al., 1997). The different biofilm behaviours may be related with the chemical reaction of the surfactants with the biofilm components used that can give rise to the strengthening or the weakening of the biofilm structure. The electrostatic bonds created stress or cross-linking depending on the chemical structure of the molecule, since CTAB is an aliphatic compound while BC is an aromatic compound. The increase in the CTAB concentration promoted the subsequent higher biofilm removal due to the destabilisation of the biofilm cohesive forces, being biofilm removal detected at a higher extent to the smaller shear stresses. Conversely, the increase in the BC concentration increased the biofilm mechanical stability face to mechanical stress conditions.

The effect of SDS on the mechanical stability of the biofilm may be due to the disruption of the hydrophobic interactions involved in cross-linking the biofilm matrix (Chen and Stewart, 2000). However, in this work, this SDS effect was only felt for the higher concentration (7 mM) tested, proposing that low concentrations of SDS can even promote the strength of the biofilm structure.

The previous application of oxidising agents improved biofilm removal by mechanical action, the effect being more pronounced with the increase in their concentration. The oxidising biocides react strongly with the EPS matrix, destroying the structure that becomes more vulnerable to hydrodynamic stress. So, it is not surprising to obtain more removal for the same $N'Re_A$ as the concentration increases.

5. Conclusions

The system presented in this work provided an approach to investigate the influence of several parameters on the mechanical stability of biofilms, leading to a better understanding of biofilms in different environments and the development of biofilm control strategies. The characterisation of the biofilms showed that the system tested allowed the formation of a great amount of biofilm that covered the surface of the SS cylinder, the biofilms being metabolically active, vastly comprising EPS and having an inherent mechanical stability.

The effect of the chemical compounds on the biofilm removal and consequent biofilm mechanical stability varied with the chemical nature; even with the synergistic chemical and mechanical treatment total biofilm eradication was not achieved in this work, for every condition studied. The application of OPA to the biofilms favoured the detachment caused by the increase in the mechanical stress, being biofilm removal similar for every concentration tested. Also, OPA demonstrated to be an alternative to GTA in the control of *P. fluorescens* biofilms, since the biofilms treated with GTA showed posterior recalcitrance properties when exposed to mechanical stress conditions, increasing with the increase of GTA concentration. The application of CTAB decreased the biofilm mechanical stability, which was more pronounced with the increase of the concentration and with the increase on the mechanical stress conditions. Conversely, BC increased the biofilm mechanical stability. This phenomenon was more pronounced with the increase of concentration. SDS caused biofilm removal due to increasing shear forces only for the highest concentration tested, when comparing with the control experiment. For the smaller concentrations, a similar effect to the one found with GTA and BC was observed. The previous application of oxidising agents (NaOH and SHC) improved biofilm removal by mechanical action, this effect being dependent on the increase in their concentrations.

This chemical diversity of agents tested (non-oxidising aldehyde-based biocides, surfactants and oxidising biocides) emphasises that multiple interactive forces contribute to biofilm mechanical stability.

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