

CONTROL OF BIOFILMS USING SURFACTANTS – PERSISTENCE AND REGROWTH

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The action of the cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS), respectively, a cationic and an anionic surfactant were investigated to control mature biofilms formed under turbulent and laminar flow, by P.fluorescens. The sanitizer action of the surfactants on biofilms was assessed by means of respiratory activity and variation of biofilm mass, immediately, 3, 7 and 12 h after the treatment of the chemicals. The latter experimental times were tested in order to assess the biofilm regrowth. The structure of the biofilms was assessed before and after surfactant treatment by SEM. The results showed that, laminar biofilms were more susceptible to the action of CTAB than those formed under turbulent flow. Concerning SDS, both biofilms showed analogous susceptibility to the surfactants. However, total inactivation of the cells within the biofilms was not achieved for both types of biofilms. CTAB application by itself did not promoted the detachment of biofilms from the surface. Regarding SDS, higher concentrations applied promoted significant biofilm inactivation. Turbulent and laminar flow had analogous susceptibility to SDS application. However, SDS did not promoted the detachment of biofilms from the metal surfaces. The structure of the biofilms was changed after the application of both surfactants. It was found that after CTAB and SDS application, the biofilms recovered its respiratory activity, reaching, in same situations, higher values than the ones found before chemical treatment. The CTAB application promoted similar recovery in the respiratory activity for both biofilms. Concerning biofilm behaviour after SDS treatment, turbulent biofilms showed a higher potential to recover their metabolic activity than laminar biofilms. Biofilm mass did not experienced any significant variation after the treatment, for both surfactants tested. This study highlights the need of care in choosing the correct procedure for biofilm control and the recalcitrant properties of biofilms.

Keywords: biofilm control, biofilm regrowth, hydrodynamic conditions, surfactant

Introduction

Biocides and disinfectants are one of the main means of controlling problems associated with microbial biofilm formation (Chen and Stewart, 2000). Surface active agents (surfactants) are commonly used in mixtures of cleaning products because of their ability of lowering surface and interfacial tensions of liquids, which comprise the ability to wet surfaces, penetrate soil and solubilize fatty materials (Christofi and Ivshina, 2002; Glover *et al.* 1999; McDonnell and Russell, 1999). Surfactants are classified on the basis of the charge or absence of ionization of the hydrophilic group namely, anionic, cationic, non-ionic and amphoteric or zwitterionic compounds (McDonnell and Russell, 1999). Cationic surfactants or quaternary ammonium compounds (QAC's) are employed also as disinfectants for manual processing lines and surfaces in the food industry, and in human medicine area (Mereghetti *et al.* 2000), because of their excellent hard-surface cleaning, deodorization and antimicrobial properties (McDonnell and Russell, 1999). QAC's mode of action is attributed to their positive charge, which forms an electrostatic bond with negatively charged sites on microorganism cell walls (McDonnell and Russell, 1999). Those electrostatic bonds create stresses in the wall, leading to cell lysis and death. The QAC's also cause cell death by protein denaturation, distortion of cell-wall permeability and reduction of the normal intake of life-sustaining nutrients to the cell (Cloete *et al.* 1998). CTAB is a QAC that appears to rupture the cell membrane. The primary site of action of CTAB has been suggested to be the lipid components of the membrane being cell lyses a second effect (Gilbert *et al.* 2002). Anionic surfactants possess strong detergent but weak antimicrobial properties, except at high concentrations, when they induce lyses of Gram-negative bacteria (Glover *et al.* 1999). The outer and cytoplasmic membranes and the membrane-bound enzyme environment and function are the main targets of anionic surfactants

(Cloete *et al.* 1998). SDS is an anionic surfactant widely used in detergent formulations (Jěrábková *et al.* 1999).

The purpose of this work was to assess the efficacy of CTAB and SDS in the sanitation of biofilms of *P.fluorescens* formed under turbulent and laminar flow, and to evaluate the capability of the biofilms to regrowth after chemical treatment.

Material and methods

Microorganism and culture conditions

Pseudomonas fluorescens (ATCC 13525^T), a Gram-negative aerobic bacteria, was used through this work. These bacteria are good biofilm producers and a major microorganism in biofilms found in industry (Pereira *et al.* 2001). Their growth conditions were 27 °C, pH 7, and glucose as the carbon source.

The bacterial culture was grown in a 0.5 l chemostat aerated and agitated with a magnetic stirrer, and continuously fed at a flow rate of 10 ml h⁻¹ with a sterile concentrated nutrient solution consisting of 5 g glucose l⁻¹, 2.5 g peptone l⁻¹ and 1.25 g yeast extract l⁻¹, in phosphate buffer at pH 7. This culture was used to continuously inoculate a 3.5 l reactor also aerated and agitated. This last reactor was fed with a minimal nutrients medium, consisting of 0.05 g glucose l⁻¹, 0.025 g peptone l⁻¹ and 0.0125 g yeast extract l⁻¹ in phosphate buffer pH 7, at a flow rate of 1.7 l h⁻¹. The bacterial suspension was pumped up, passing through the flow cell reactors described elsewhere (Pereira *et al.* 2002a) and back to the 3.5 l reactor.

Surfactants

Two surfactants were tested:

Cetyltrimethyl ammonium bromide – CTAB (Merck, critical micellar concentration (CMC) – 1.00 mM; Cat. No. 102342) at 0.125, 0.250, 0.500 and 0.900 mM.

Sodium dodecyl sulfate – SDS (Riedel-de-Haën, CMC – 8.30 mM; Cat. No. 62862) at 0.500, 1, 3 and 7 mM.

Surfactant solutions were diluted to the required concentration with sterile water.

Biofilm system

A continuous flow cell reactor described by Pereira *et al.* (2002a) was used for biofilm. It consists of a semi-circular PMMA duct with several apertures on its flat face to fit several coupons where biofilm formation surfaces (1.75 cm × 1.25 cm) were glued. These surfaces, which in the case under study were ASI 316 stainless steel slides (SS), were in contact with the fluid circulating in the system. Biofilms were formed by recirculating the bacterial suspension, obtained from the 3.5 l reactor, through two similar flow cell reactors operating in parallel, each one with ten slides for biofilm sampling. One of the flow cells was used to promote laminar flow ($Re = 2000$, $u = 0.204 \text{ m s}^{-1}$) and the other turbulent flow ($Re = 5200$, $u = 0.532 \text{ m s}^{-1}$). The biofilms were allowed to grow for 7 d to ensure that steady-state biofilms were used in every experiment (Pereira *et al.* 2002a).

Biofilm tests

The biofilms formed on the metal slides of each parallel flow cell reactor were exposed to different concentrations of surfactant for ½ h. Each surfactant concentration was tested in an independent experiment and each experiment was performed on three separate occasions. During the treatment period (½ h), the surfactant solution replaced the diluted bacterial suspension flowing in the flow cell reactors. After the exposure time to the surfactant, the flow of the surfactant solution through the flow cells was stopped and the bacterial suspension was re-introduced in the system. In each experiment, and prior to the beginning of the surfactant treatment, two metal slides of each flow cell

were sampled and used as a control. Immediately after the ½ h surfactant treatment, two metal slides of each flow cell were also sampled (time zero). The biofilms that covered the SS slides were completely scraped, resuspended in 10 ml of a neutralization solution - 0.1 % (w v⁻¹) peptone, 0.5 % Tween 80 and 0.07 % lecithin, dissolved in phosphate buffer pH 7 - and left during 10 min. After the neutralization step, the biofilm suspensions were vortexed during 30 s with 100 % input, and then washed two times with saline phosphate buffer, resuspended in phosphate buffer and used immediately to assess the bacterial activity of the biofilm through oxygen uptake rate. Afterwards, the suspension was used to determine the biofilm mass. In order to assess whether time plays a significant role on the action of surfactant, namely if it prevents a subsequent growth of the biofilm, the remaining slides were left in the flow cells and were sampled 3, 7 and 12 h after surfactant application. For every condition tested and for all times of exposure, two SS slides were sampled.

Analytical Methods

Biofilm Mass

The dry mass of the biofilm accumulated on the slides was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions, according to the Standard Methods (APHA, AWWA, WPCF, 1989), method number 2540 A-D. The biofilm mass accumulated was expressed in mg of biofilm *per* cm² of surface area of the slide (mg_{biofilm} cm⁻²). In each experiment, the percentage of the biofilm removal was determined through the following equation:

$$\text{Biofilm removal (\%)} = [(W - W_1) W^{-1}] \times 100 \quad (1)$$

where W is the biofilm mass without surfactant application (mg_{biofilm} cm⁻²) and W_1 is the biofilm mass after surfactant treatment (mg_{biofilm} cm⁻²).

Respiratory activity assessment

The respiratory activity of the several samples was evaluated by measuring the oxygen uptake rate needed to oxidise glucose in a biological oxygen monitor (BOM) in short-term assays. The assays were performed in a Yellow Springs Instruments BOM (Model 53) and the procedure used was described elsewhere (Simões *et al.* 2003b).

The decrease in the bacterial activity obtained due to the application of the different concentrations of surfactant to both bacterial biofilms was determined as the difference between the respiratory activities of the samples before (control) and immediately after the treatment period with surfactant, and expressed as the percentage of inactivation according to the following equation:

$$\text{Inactivation (\%)} = [(A_0 - A_1) A_0^{-1}] \times 100 \quad (2)$$

where A_0 is the respiratory activity of the control assay, *i.e.*, without surfactant treatment ($\text{mg O}_2 \text{ g}_{\text{biofilm}} \cdot \text{min}^{-1}$), and A_1 is the respiratory activity immediately after the application of each surfactant concentration ($\text{mg O}_2 \text{ g}_{\text{biofilm}} \cdot \text{min}^{-1}$).

Scanning electron microscopy observations

Scanning electron microscopy (SEM) inspections were performed according to the procedure described by Simões *et al.* (2003b).

Statistical analysis

The mean and standard deviation within samples were calculated for all cases. Statistical comparisons of biofilm inactivation, biofilm removal and regrowth were analysed by *t* Student's test.

Results and discussion

Biofilm inactivation and removal after CTAB application

The effects of the application of CTAB during ½ h against *P. fluorescens* biofilms formed on SS slides, under turbulent and laminar flow was assessed either by determining the respiratory activity

due to glucose oxidation and the variation of the mass of biofilm. Those results are presented in terms of percentage of biofilm inactivation and removal (Figure 1), immediately after CTAB application.

—————→ Figure 1

Previous studies (Simões *et al.* 2003a) reported that the specific respiratory activity and mass of biofilms formed under turbulent flow is higher than biofilms formed under laminar flow. Turbulent biofilms were about five times more active and had about two times more mass than laminar biofilms. The application of CTAB to biofilms formed in the flow cell reactors resulted in an inactivation of the bacteria within the biofilm, which increased with the increase of the surfactant concentration (Figure 1a). Concerning the studies carried out with biofilms formed under different flow regimes, the inactivation effect of CTAB was more pronounced in laminar biofilms than turbulent biofilms ($P < 0.05$). Nevertheless, total biofilm inactivation was not achieved for every condition studied. From these results obtained, it can be said that the development of successful strategies to control biofilm formation must be studied under conditions that mimic real situations, since biofilm properties change in response to environmental conditions (Pereira *et al.* 2002b; Vieira *et al.* 1993). The understanding of the effect of operational parameters in biofilm formation and subsequent disinfection plays a basic role on the establishment of a biofilm control program. Previous studies made by some authors (Pereira *et al.*, 2002b; Vieira *et al.* 1993), concerning the characterisation of biofilms formed under turbulent and laminar flow, showed that biofilms formed under turbulent flow were more active and had a higher content of proteins than laminar biofilms and that their physical structure was different. In the present study, the low efficacy of CTAB to control biofilms may be related with its chemical reaction with proteins of the exopolymeric matrix. This argument is reinforced by the tests carried out with planktonic cells, which showed that the inactivation effect of CTAB was significantly reduced in the presence of BSA (Simões *et al.* 2005). The higher inactivation effect on laminar biofilms is probably related with the less amount of biofilm formed, compared with turbulent biofilms (Simões *et al.*, 2003a), and, consequently, to the

less content of proteins which increases the CTAB available for reaction with the cells. In both hydrodynamic situations, problems associated with mass transfer limitations within the biofilms can, always, decrease the action of CTAB.

Concerning biofilm removal (Figure 1a), CTAB had not a significant effect, since the biofilm removal was always less and similar than 25 %, independently of the CTAB concentration. For biofilms formed under laminar flow, the higher detachment was induced by a concentration of 0.250 mM, while for turbulent biofilms it was achieved only for a concentration of 0.5 mM.

Comparing statistically the percentage of biofilm removal for turbulent and laminar biofilms the results are similar ($P > 0.1$).

Concerning the comparison of the results of inactivation and removal, CTAB showed higher ability to inactivate the biofilm than in remove it from surfaces, leaving biofilm on the surface not fully inactivated. Azeredo *et al.* (2003) already showed that CTAB (0.5 mM) had the ability to cement bacteria to glass in spite of removing them.

Biofilm inactivation and removal after SDS application

Results of biofilm respiratory inactivation and biofilm removal after treatment with SDS at several concentrations are plotted in Figure 2.

—————→ Figure 2

SDS promoted biofilm inactivation, being this effect dependent of the concentration (Figure 2a). However, in the range of concentrations tested, total inactivation was not achieved, as found for CTAB. Comparing the results obtained for the turbulent and laminar biofilms, the statistical analysis showed that both biofilms had similar susceptibility to SDS action ($P > 0.1$). The reaction of the surfactant, as suggested for CTAB, with the constituents of the biofilm seems to be again the phenomenon behind the inefficiency of SDS to promote total biofilm inactivation. Concerning biofilm removal (Figure 2b), SDS had a poor effect on the biofilm removal, for both biofilms, since in almost experiments the biofilm removal was less than 20 %. However, Azeredo *et al.* (2003)

used SDS to remove efficiently monolayer of cells adhered to glass. In this work besides the small amount of biofilm removed by SDS, removal was not dependent with the surfactant concentration, since the increase in the SDS concentration did not increased biofilm removal. The statistical analysis revealed no equivalence on the removal of biofilms formed under different flow regimes ($P < 0.05$). This difference found within biofilms reflects the impact of the flow regime under which the biofilm are formed in the posterior biofilm removal. In fact, Purevdorj *et al.* (2001) found that high shear flow leads to a formation of strongly *P. aeruginosa* adhered biofilms. However, the results presented so far underscore the fact that biofilm inactivation and biofilm removal are distinct processes. The permanence of a remaining pellicle on a surface that is still active, or in another metabolic state, may be a source of problems, such as biofilm regrowth, development of resistant biofilms or an additional substrate for other microorganisms.

Structural changes due to surfactant application

The evidence of bacterial biofilm in the metal slides before the treatment and the possible damage resulting from surfactant treatment was inspected by SEM, as displayed in Figure 3.

—————→ Figure 3

Despite the low effect on the biofilm removal, SEM observations reveal that the biofilm structure is changed after CTAB application (Figure 3). Biofilms formed under different flow regimes present morphological differences, and that CTAB reacts with the components of the biofilm, since after treatment with 0.5 mM of CTAB the structure of the biofilm is altered. Concerning SDS treatment, it seemed to cause damage in the structure of the bacterial biofilms. The probable phenomenon behind this fact is related with reaction of SDS with the biofilm cells and the removal of layers of biofilm. The treatment of laminar biofilms with 3 mM of SDS gave rise to a clear reduced amount of biofilm, which is in accordance with the result found for the biofilm removal, where this value is higher for this condition.

Biofilm recovery after treatment with CTAB and SDS

The results presented in Figures 1 and 2 emphasize that after ½ h of contact with the surfactants, and for all the concentrations tested, biofilms still show respiratory activity. In order to know whether this fact could lead to biofilm regrowth, the post-surfactant effect was evaluated along 12 hours. In order to avoid misleading results, appropriate control experiments (without surfactant treatment) were also carried out. Figure 4 presents the post-surfactant effect, in terms of respiratory activity, of turbulent (a) and laminar (b) biofilms, after surfactant treatment. That effect was evaluated after 3, 7 and 12 h later and compared with the results obtained immediately after the chemical treatment (0 h).

—————→ Figure 4

From Figure 4aI and 4bI, it can be seen that the respiratory activity increased with the time between CTAB application and biofilm sampling, reaching values higher than the ones observed in the control experiment, *i.e.*, without surfactant application. Both turbulent and laminar biofilms have similar regrowth profiles ($P > 0.05$). The control experiments show that the biofilm activity was almost independently of the time ($P > 0.05$) since the 7 d old biofilms exhibited the same respiratory activity during the time of experiment (12 h). This result was expected since biofilms are in a metabolic steady-state (Pereira *et al.* 2002b).

From the results obtained after treatment with SDS (Figure 4aII and 4bII), the activity of biofilms increased with time, particularly when 3 mM and 7 mM of SDS were applied to the biofilms. However, for turbulent biofilms the regrowth was more pronounced than for laminar biofilms ($P < 0.05$). Also, for turbulent biofilms, after SDS application, the regrowth was more pronounced with the increase of the SDS concentration applied.

Comparing the results of biofilm regrowth for both surfactants, the regrowth is more evident for biofilms treated with CTAB and less clear to laminar biofilms treated with SDS (Figure 5). The ionic nature of the surfactant seems to be responsible for the alteration effects of the biofilm respiratory activity, playing a more significant action when the surfactant concentrations applied

were near the CMC. Consequently, the biofilm regrowth must be associated with the stress conferred by the surfactant application. Probably, the surfactant may have increased the availability of nutrients to the cells within the biofilms (promoting bacterial recovery) since the surfactant have changed the structure of the biofilm matrix as inspected by the SEM results, favouring nutrient diffusion inside the matrix. Another feature that could contribute to the biofilm regrowth was the pre-establishment of the operational conditions verified prior surfactant application, as the supply of nutrients. Similar suggestion was pointed out by Chandy and Angles (2001) where they found that one of the key factors that determine bacterial recovery in drinking water distribution systems is the availability of nutrients. Additionally, the bacteria found within the biofilms can present changes in their metabolic steady-state. In some cases, with surfactant application, this metabolic state seems to turn into a state of higher metabolic activity, different from the one found for the control experiment. This preservative recovery, according to Stewart (2003) could lead to populations of resistant bacteria, which may be recalcitrant to a subsequent disinfection process. The overall results suggested that if the biofilms were left more time in the flow cell reactors, probably, the recovery of biofilm would be more evident and consistent. Furthermore, the biofilms were stained with Live/Dead *BacLight* kit (results not shown) before and after surfactant treatment (during the 12 h of experiment), showing that the biofilm left on the flow cell after surfactant treatment recovered their viability during the 12 h of the experiment, corroborating the respiratory activity results (Figure 4).

The dry biofilm mass before and after surfactant application can be observed in Figure 5.

—————→ Figure 5

It can be seen that, in terms of total biofilm mass, the application of CTAB to both turbulent and laminar biofilms (Figures 5aI and 5bI) did not gave rise to biomass decrease. On the contrary, it seems that the application of CTAB increased the amount of biofilm adhered to the SS slides. Concerning SDS (Figures 5aII and 5bII), only small variations were achieved with the surfactant treatment, being those variations more noticeable for laminar biofilms. Therefore, it is clear that

the application of SDS or CTAB and the time did not promoted any significant additional biofilm removal or biofilm growth, for any conditions tested and for any sampling time ($P > 0.05$, for both surfactants and for every condition tested).

It must be emphasized that the biofilms which were not immediately sampled after surfactant application were not subjected to the neutralization step. So it was expected a sustained antimicrobial effect that promoted the failure of the cohesive forces of the biofilm, encouraging the consequent removal, since the surfactant retained within the biofilm matrix had more chance to act on the bacteria. Forsyth and Hayes (1998) stated that surfaces treated with cationic surfactants could retain a bacteriostatic film, due to the adsorption of the chemical on the surface, that could prevent the subsequent growth of residual bacteria. However, the data presented in this study proved that the surfactant did not induce suppression of biofilm recovery in terms of biofilms activity and did not promote gradual biofilm erosion for both biofilms.

Conclusions

A better understanding of biofilm response face to an external stress condition is essential for the emerge of efficient new strategies for controlling biofilms. Biofilms formed under laminar flow were more susceptibly to the surfactant inactivation effect than turbulent biofilms, but none of them were removed by the surfactants tested. A post-surfactant effect was noticed for both biofilms since they gradually recovered their metabolic activity, after surfactant treatment. Concerning biofilm mass, surfactants did not promote a slow biofilm detachment or the increase in the biofilm mass, probably, due to the limited time of experiment.

This improvement in the understanding of the relationship between surfactant molecular properties and antibacterial properties and mechanisms of action could facilitate the design of chemical mixtures that more effectively control biofilm activity and removal.

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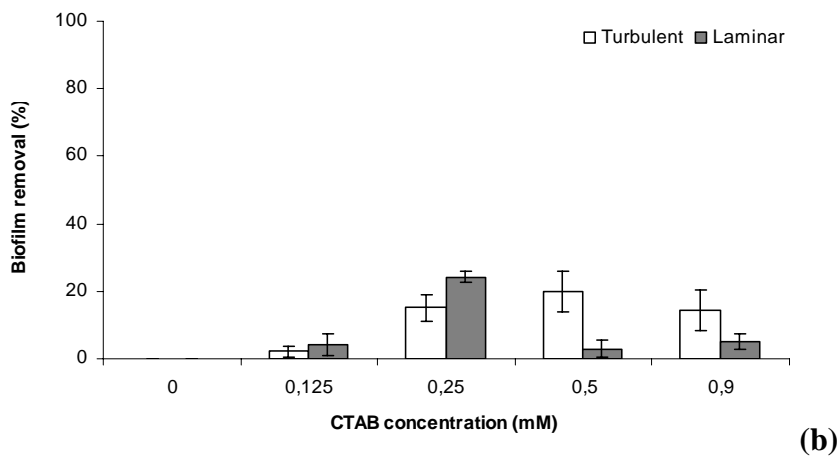
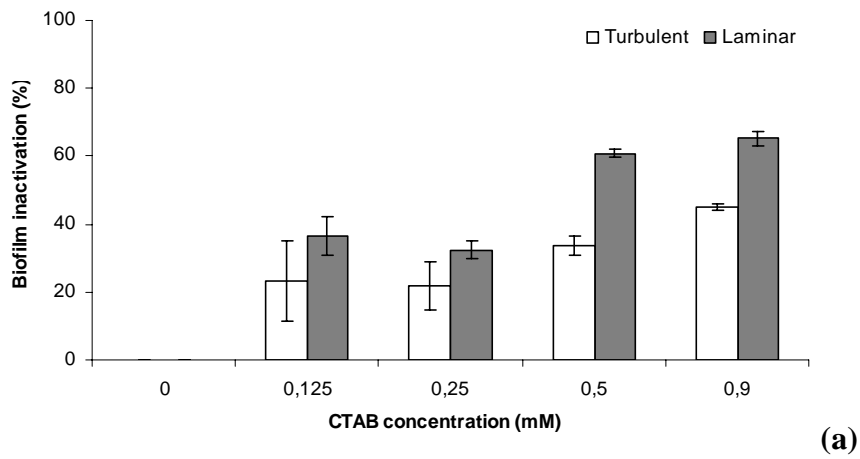


Figure 1 Biofilm inactivation (a) and removal (b) due to application of different concentrations of CTAB. Each bar indicates the means \pm SD.

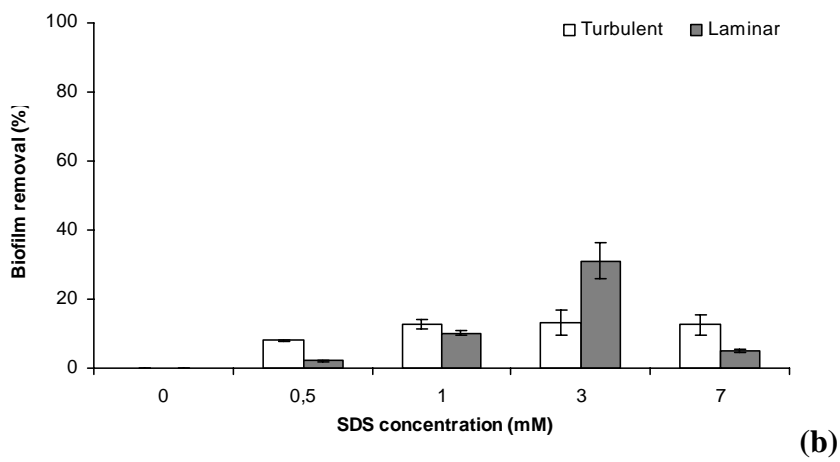
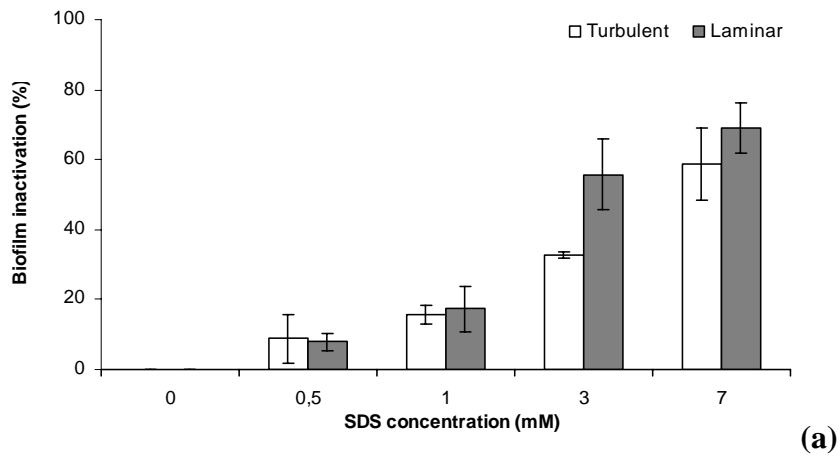


Figure 2 Biofilm inactivation (a) and removal (b) as a function of SDS concentration. Each bar indicates the means \pm SD.

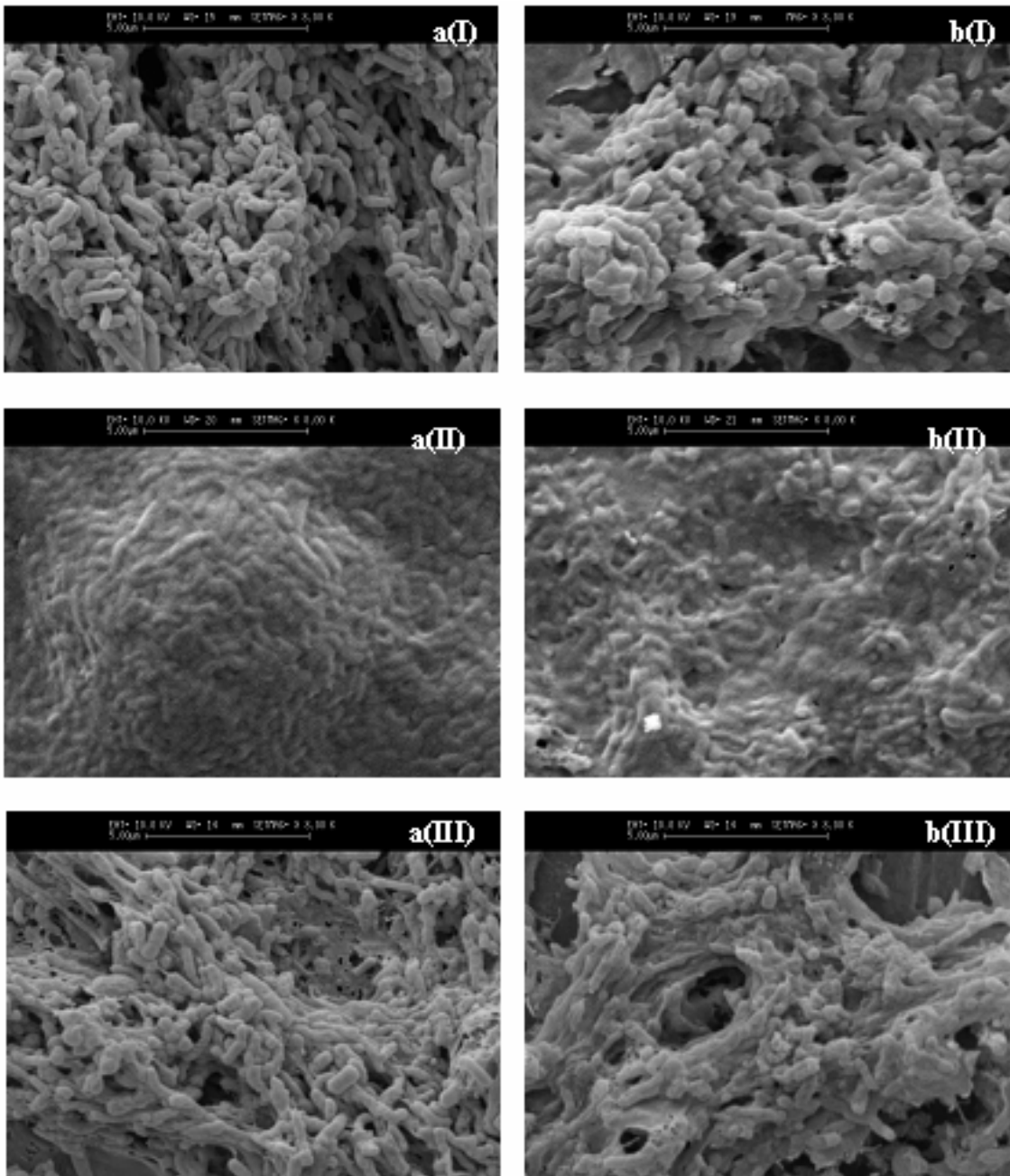


Figure 3 SEM microphotographs of a 7 d old *P. fluorescens* biofilms formed on the SS slides under turbulent (a) and laminar flow (b) without surfactant application (I), after treatment with 0.5 mM of CTAB (II) and after treatment with 3 mM of SDS (III) during 30 min. X 8000 magnification, bar = 5 µm.

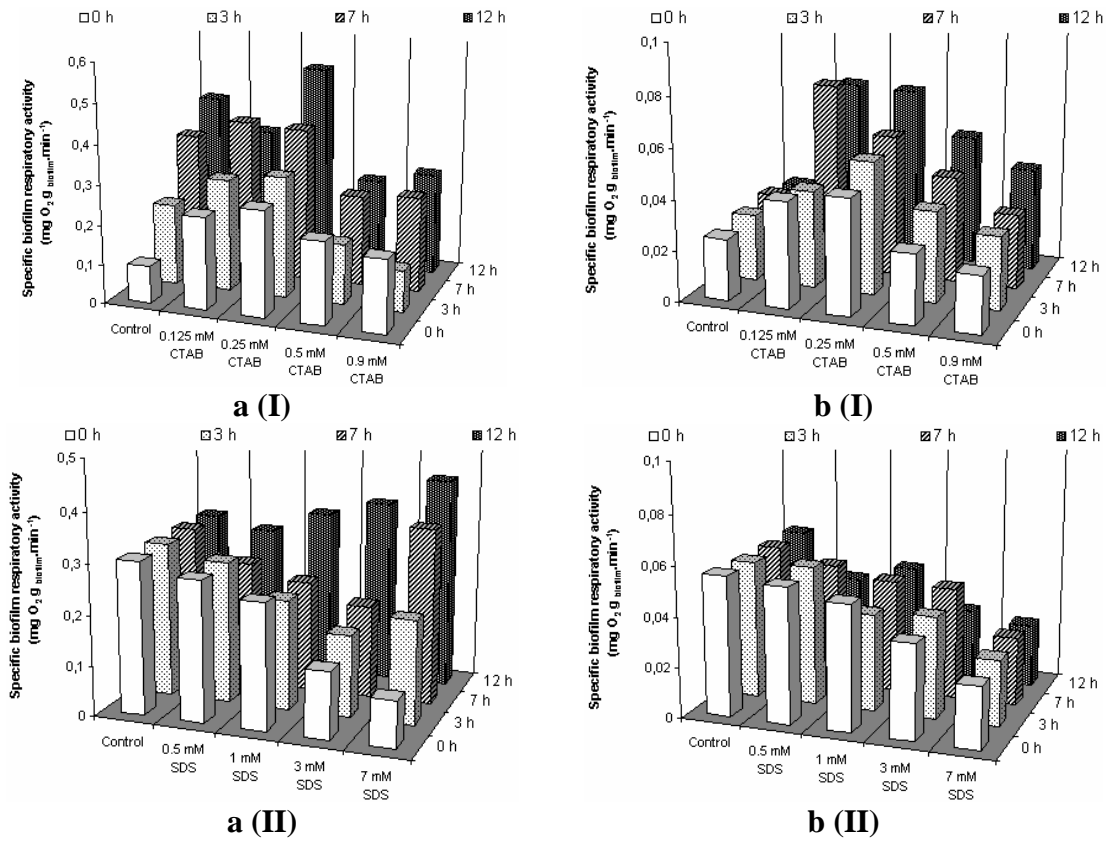
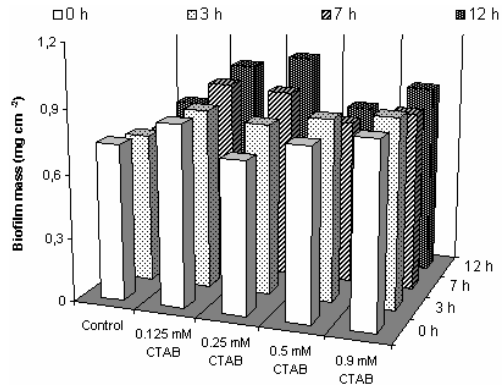
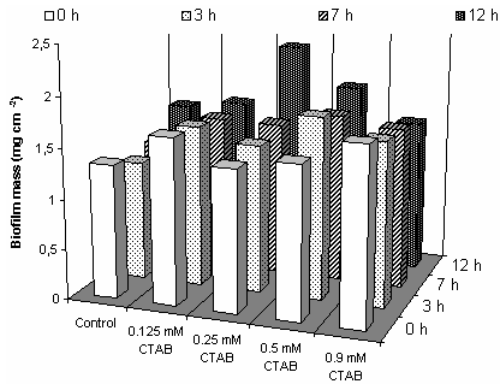
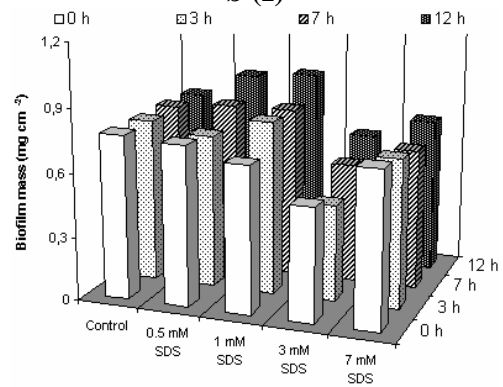
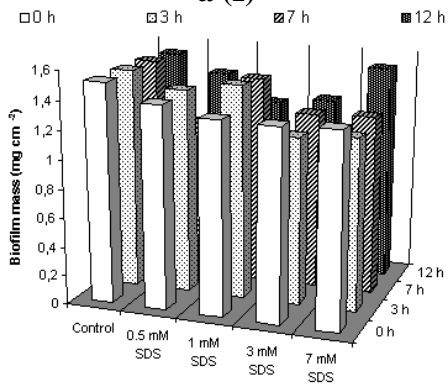


Figure 4 Biofilm respiratory activity after treatment (0 h) and 3, 7 and 12 h later with CTAB (I) and SDS (II) for biofilms formed under turbulent (a) and laminar (b) flow.



a (I)

b (I)



a (II)

b (II)

Figure 5 Biofilm mass after chemical treatment (0 h) and 3, 7 and 12 h later with CTAB (I) and SDS (II) for biofilms formed under turbulent (a) and laminar (b) flow.