

Effect of cationic surfactants on biofilm removal and mechanical stability

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Abstract

In this study, a methodology is proposed to evaluate the mechanical stability of biofilms, by using a stainless steel rotating device where biofilms formed by *Pseudomonas fluorescens* were allowed to grow for 7 days at 300 rpm. Those biofilms were afterwards submitted to the joined action of chemical agents and mechanical cleaning. Two cationic surfactants (cetyltrimethyl ammonium bromide – CTAB and benzalkonium chloride – BC) were the agents tested. Mechanical cleaning was performed by the variation of the rotation speeds of the rotating device. The following conclusions can be drawn: the biofilm has an inherent mechanical stability; the increase in the rotation speed increased the biofilm removal, but total biofilm removal was not found for the surfactants. BC promoted the increase in the biofilm mechanical stability while CTAB promoted the weakening in the biofilm mechanical stability. From this study, it can be stated that the chemical treatment is far from being a tool that induces massive biofilm detachment and even the synergistic chemical and mechanical treatment did not promoted total biofilm removal.

Keywords

Biofilm behaviour; biofilm control; biofilm stability; chemical control

INTRODUCTION

Biofilms consists of both microorganisms and their extracellular polymeric substances (EPS) attached to a surface. Once developed, biofilms are difficult to remove completely (Simões *et al.*, 2003). Chemical agents and mechanical forces are the main tools involved in biofilm removal, despite of mechanical cleaning being often unfeasible (Srinivasan *et al.*, 1995). Thus, appropriate selection and application of chemical agents should be made in order to obtain successful biofilm control. Furthermore, these chemical agents tend to leave the biofilm intact when no mechanical treatment is involved in the process (Flemming, 1996). The EPS provided the biofilm mechanical stability by filling and forming the space between the cells, keeping them together (Körstgens *et al.*, 2001). 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 or control of biofilms in engineered systems (Poppele and Hozalski, 2003). So far, very limited studies have been done regarding the mechanical stability of biofilms (Körstgens *et al.*, 2001; Poppele and Hozalski, 2003; Stoodley *et al.*, 1999), as well as any study concerning the effect of chemical agents on that parameter was reported.

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

MATERIALS AND METHODS

Microorganism and Culture conditions

Pseudomonas fluorescens (ATCC 13525^T) was grown in a 0.5 L reactor aerated, agitated, and continuously fed (10 mL h⁻¹) with growth media 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 (PB).

Biofilm reactor system and operation

Biofilms were grown for 7 days on ASI 316 stainless steel (SS) cylinders (surface area of 34.6 cm²) inserted in a 3.5 L reactor. This reactor was continuously inoculated with the bacterial culture (coming from the 0,5 L reactor), and fed with a diluted nutrient medium (50 mg L⁻¹ glucose, 25 mg L⁻¹ peptone, 12.5 mg L⁻¹ yeast extract in PB).

Treatment chemicals

Cetyltrimethyl ammonium bromide – CTAB and Benzalkonium chloride – BC: two cationic surfactants, were tested throughout this work.

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). Biofilm mass was expressed in g per cm² of surface area of the metal slide.

Physical stability of the biofilm

This parameter was investigated by biomass loss after submitting the biofilms covered the SS cylinders to increasing rotating speeds. After 7 days of operation, the cylinders *plus* biofilm were removed from the reactor. One of the cylinders was immersed in a reactor with PB (control experiment) while the others were put in reactors containing the chemical solutions, during 30 min. After that, the cylinders were removed, weighed, introduced in reactors with PB, and consecutively subjected to serial velocities of rotation, *i.e.*, 500, 1000, 1500, and 2000 rpm, for a period of 30 s each. The wet weight of the cylinders *plus* biofilm attached was ascertained before and after each speed. The wet mass of the biofilm removed from the cylinder surface, after each rotation speed, was expressed in % of biofilm removal, and the biofilm that stayed adhered on the cylinders after all the rotation speeds was expressed as % of biofilm remaining.

Statistical analysis

The mean and standard deviation within samples were calculated for all cases. The statistical software package SPSS was used for statistical analysis. The results were statistically tested by using a parametric test (one-way analysis) when data were normally distributed as assessed by the Levene's test of homogeneity. Nonparametric statistical tests (Mann-Whitney or Krustal-Wallis) were used for data that were not normally distributed. Statistical calculations were based on confidence level equal or higher than 95%.

RESULTS

Figure 1 shows a SS cylinder covered with biofilm after 7 days of growth.



Figure1. Stainless steel cylinder covered with biofilm.

It can be noticed that the amount of *P. fluorescens* biofilms adhered to the cylinder was very significant, being the cylinder surface totally covered with biomass. The EPS matrix of the biofilms was also well noticeable by this Figure.

Figure 2 shows the biofilm removal obtained due to the increase of the cylinder rotation speed, for the control experiment (without chemical treatment) and for the treatments with several concentrations of CTAB and BC. The application of shear stress forces higher than the one under which the biofilm was formed gave rise to some biofilm removal. The higher percentage of removal occurred with the application of 1000 rpm, being the % of removal similar for the others rotation speeds. So, it can be stated that biofilm removal is dependent on the rotation speed implemented ($P < 0.05$). Figure 2 shows, also, that the biofilm has an inherent mechanical stability since, in the control experiment, the biofilm remaining, after submission to the total series of rotation, 24% of the biofilm mass was still attached to the cylinder surface. The joined action of chemical application and shear forces change (due to the variation of the rotational velocity) resulted in biofilm remaining ranged from 3% - for 0.900 mM of BC - to 47% - for 0.900 mM of CTAB. CTAB increased the removal of the biofilm of the cylinders, since the biofilm mass that remained on the cylinder surface decreased with the chemical treatment, in opposition to the effect promoted by BC. Moreover, that decrease was more evident with the increase of the chemical concentration applied. Conversely, the amount of biofilm that remained on the cylinders increased with the BC concentration tested. This fact suggests that BC, in despite of being also a cationic surfactant as CTAB, contributes, in some way, to a higher biofilm mechanical stability.

Comparing the biofilm removal for the several rotation speeds applied, for the smaller ones, the increase on the BC concentration tends to promote a decrease on the biofilm removal. However, biofilm removal is equivalent ($P > 0.10$) for the same rotation speed when comparing the different BC concentrations. Only with the application of 0.900 mM of BC significant differences ($P < 0.05$) in biofilm removal were found for the different rotation speeds, being the higher amount of biofilm removal (30%) observed for 2000 rpm.

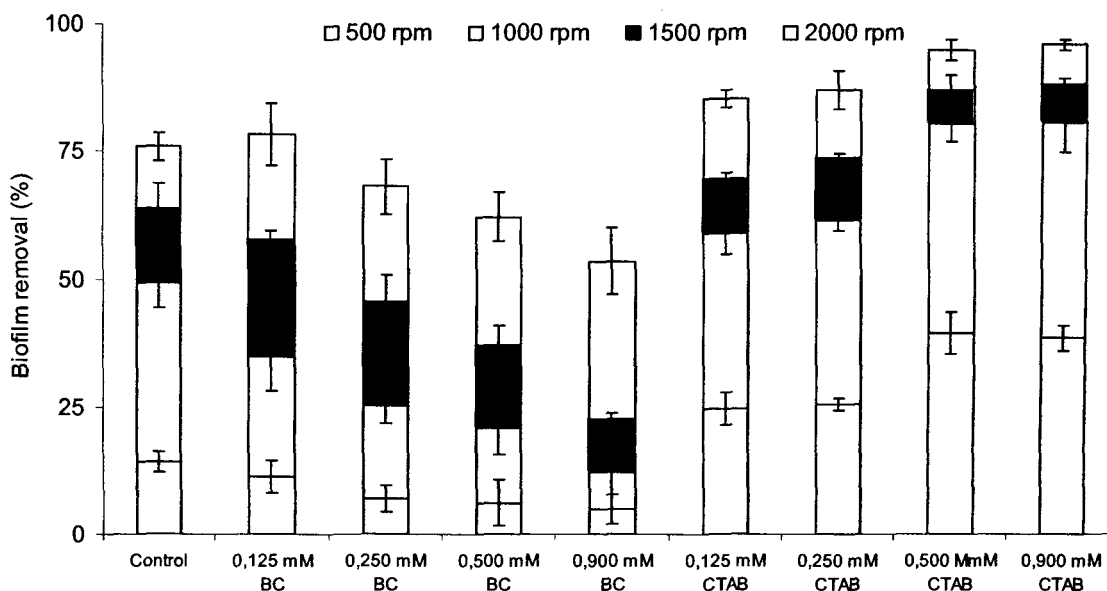


Figure 2. Biofilm removal observed after the implementation of each rotation speed for the control experiment and following biofilm exposure to several concentrations of BC and CTAB.

DISCUSSION

The high content of EPS found in the biofilms, as demonstrated by Figure 1, is, according to Körstgens *et al.* (2001), responsible for keeping biofilm together and forming a temporary network of fluctuating junction points. The EPS strengthened the cohesive forces within the biofilm, thereby contributing to a greater biofilm mechanical stability (Azeredo and Oliveira, 2000). To remove a well established biofilm it is need to overcome the forces which maintain the integrity of the biofilm matrix, as well as, the forces that keep the biofilm adhered to the support (Körstgens *et al.*, 2001). The control experiment shows that biofilms subjected to sole mechanical treatment, were hardly removed with low rotations speeds (≤ 500 rpm) since only about 14 % of biofilm removal was achieved. However, biofilm removal increased with the increase in the rotation speed. According to Azeredo and Oliveira (2000), this biofilm detachment is processed in layers where the high rotation speeds progressively thin the biofilm. These results suggest that biofilm mechanical stability and consequent biofilm removal seem to be phenomena more related with mechanical action rather than with chemical action. Nevertheless, the most common practice to eliminate biofilms involves the use of antimicrobial chemicals (Chen and Stewart, 2000). In order to see whether these chemicals could play an additional role in biofilm removal, the coupled action of chemical treatment and variation of shear forces was investigated. The treatments with the surfactants caused different biofilm responses that may be related with the chemical structure of the surfactant, by strengthening or weakening the biofilm structure. The action of surfactants is attributed to their positive charge that forms an electrostatic bond with negatively charged sites (Clöete *et al.*, 1998). The electrostatic bonds created stress or cross-linking depending on the chemical structure since CTAB is an aliphatic compound while BC is an aromatic compound. The increase in the CTAB concentration promoted the posterior higher biofilm removal, while the increase in the BC concentration increases the biofilm mechanical stability giving rise to the formation of a persistent deposit that remained in the surface after treatment.

CONCLUSIONS

The system described in this work provided an interesting approach to investigate the influence of some parameters on the mechanical stability of biofilms, leading to a better understanding of biofilms in different environments and the development of biofilm control strategies. It can be concluded that the effect of the surfactants on biofilm removal varied with the chemical characteristics and that chemical treatment is far from being a cause that induces massive detachment. Even with the synergistic chemical and mechanical treatment total biofilm eradication was not achieved in this work. The results emphasize that care must be taken and useful experiments must be made in order to the choose of the correct protocol for biofilm control since chemicals can strengthen biofilm structure and thus decrease biofilm removal, leading to the increase of the biofilm mechanical stability.

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