Cellular changes due to biofilm formation – influence of flow regime

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Abstract
A comparison of the biochemical and metabolic characteristics of *Pseudomonas fluorescens* in planktonic state and cells developed in biofilms is presented in this study. The effects of the flow regime (turbulent and laminar) under which the biofilms were formed were also studied. The comparative study was performed by assessing the respiratory activity, the cell number and the amount of cellular proteins and polysaccharides. The results obtained showed that planktonic cells were more active, and had a higher content of proteins and polysaccharides per cell than the cells present in biofilms. Nevertheless, the number of cells per mg of biological mass is much higher in biofilms. Concerning the comparison between biofilms formed under different flow regimes, the ones formed under turbulent flow were more active and had a higher number of cells than biofilms formed under laminar flow. The intracellular proteins and polysaccharides are in a higher extent in biofilms formed under laminar flow. Furthermore, SEM photomicrographs show that biofilms formed under different flow regimes also look very differently.

Keywords
Biofilm behaviour; hydrodynamic conditions; phenotypic changes; planktonic cells

INTRODUCTION

A better understanding of biofilm behaviour is particularly important because of the many problems associated with biofilm formation and control, ranging from medical infections to the fouling of industrial components (Stoodley *et al.,* 1999). It is known that surfaces promote novel behaviour in bacterial cells since the physiological changes in the transition from planktonic to attached cells are profound and complex (Sauer and Camper, 2001; Sauer *et al.,* 2002). Concerning biofilm development, one of the most important factors affecting biofilm structure and activity is the velocity of the fluid in contact with the attached microbial layer (Pereira *et al.,* 2002b).
This work provides a metabolic and a biochemical comparative study between cells in planktonic and in cells embedded biofilm. It was also analysed the influence of the flow regime in the biofilm characteristics.

MATERIAL AND METHODS

Microorganism

Pseudomonas fluorescens ATCC 13525^T was used throughout this work.

Experiments with planktonic bacteria

Microorganism growth
A continuous pure culture of the *Pseudomonas fluorescens* was grown in a 2 L glass reactor, at 27 °C, suitably aerated and magnetically agitated. The reactor was continuously fed with 0.05 L h^{-1} of sterile medium containing 5 g L^{-1} glucose, 2.5 g L^{-1} peptone and 1.25 g L^{-1} yeast extract in phosphate buffer pH 7. Periodically, aliquots of *Pseudomonas fluorescens* were sampled from the reactor, centrifuged (3777*g*, 5 min) and washed three times with saline (NaCl 0.85 %) phosphate buffer pH 7 to immediately assess cellular respiratory activity and thereafter, to determine the amount of cellular proteins and polysaccharides, the cellular number and the mass of bacteria.

Experiments with biofilms – Flow cell reactor
A flow cell described elsewhere (Pereira *et al.*, 2002a) was used to develop bacterial biofilms on ASI 316 stainless steel (SS) slides. Two flow cells were operating in parallel, in order to be possible to obtain one cell working under laminar flow (Re=2000, u=0.204 m s^{-1}) and the other cell under turbulent flow (Re=5200, u=0.532 m s^{-1}). The biofilm was allowed to grow for 7 days. Biofilm development and posterior scrapping and disagregation was performed as described by Simões *et al.* (2003).

Oxygen uptake rate
The respiratory activity of the several samples was evaluated by measuring oxygen uptake rates in a biological oxygen monitor (BOM) in short-term assays. The assays were performed in a Yellow Springs BOM (Model 53) and the procedure used was described elsewhere (Pereira *et al.*, 1998).

Extraction procedure
After the respiratory activity assessement, the extraction of the extracellular components of the biofilm and the planktonic cells was carried out using Dowex resin (50X 8, NA^+ form, 20-50 mesh, Aldrich-Fluka 44445) according to the procedure described by Frolund *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 1mM KCl, pH 7). The biological samples were ressuspended in 20 ml of extraction buffer and 50 g of Dowex resin per g of volatile solids were added to the biofilm and planktonic cells and the extraction took place at 400 rpm for 4h at 4 °C. The extracellular components were separated from the cells with a centrifugation (3777*g*, 5 min).

Analytical methods
The cellular proteins were determined using the Lowry modified method (SIGMA-Protein Kit n° P5656) and the cellular polysaccharides by the phenol-sulphuric acid method of Dubois *et al.* (1956).

Quantification of the number of cells
After the extraction procedure the cells separated from the extracellular products were diluted to an adequate concentration, being thereafter stained with DAPI as described by Saby *et al.* (1997).

Biofilm and planktonic cells mass quantification
The dry planktonic cell mass and the biofilm cell mass were assessed both by the determination of the total volatile solids (TVS) according to the APHA, AWWA, WPCF Standard Methods (1989).

Scanning electron microscopy (SEM) observations
During the experiments, several stainless steel slides covered with biofilms were observed by SEM. The SEM inspections always comprised the observation of at least 15 fields of each biofilm-covered slide. Prior to SEM observations, the biofilm samples were gradually dehydrated in an absolute ethanol series to 100% (15 min each in 10, 25, 40, 50, 70, 80, 90 and 100% v/v), and dried in a desiccator for 3 d. The samples were sputter-coated with gold and examined with a Leica S360 scanning electron microscope at 10-15kV. The slides were not fixed because fixation procedures involves the use of chemicals that tend to react with some of the components at the biological matrix, as documented by Azeredo *et al.* (1999), hence modifying the real biofilm structure. SEM observations were documented through the acquisition of representative microphotographs.

RESULTS

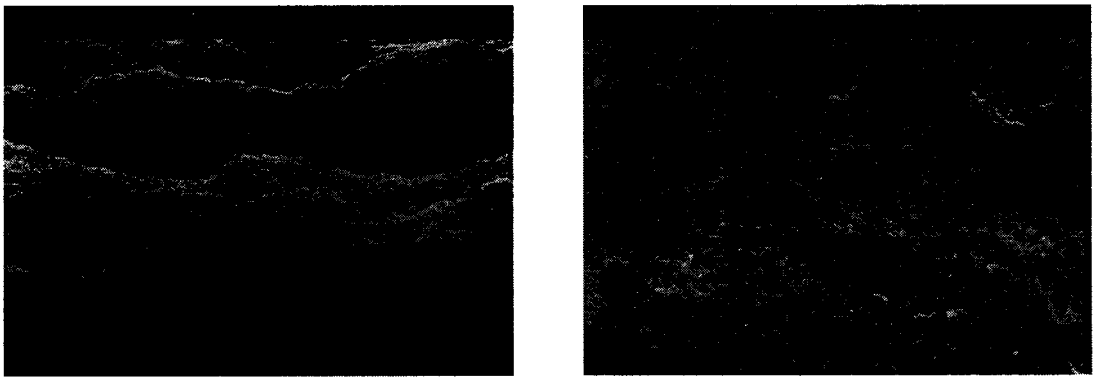
With the aim to compare planktonic cells and cells within biofilms formed under turbulent and laminar flow, ratios of respiratory activity, cell number and the amount of intracellular proteins and polysaccharides are presented in Table 1.

Table 1. Comparative ratios of respiratory activity (RA), cell number (CN), and cellular proteins (CPT) and polysaccharides (CPL), between planktonic cells and cells in biofilms formed under turbulent and laminar flow.

RA Planktonic/RA Turbulent biofilm (mgO_2/g cells.min)/(mgO_2/g cells.min)	1.63
RA Planktonic/RA Laminar biofilm (mgO_2/g cells.min)/(mgO_2/g cells.min)	8.27
RA Turbulent biofilm/RA Laminar biofilm (mgO_2/g cells.min)/(mgO_2/g cells.min)	4.70
CN Turbulent biofilm/CN Planktonic (cells/mg cells)/(cells/mg cells)	2145
CN Laminar biofilm/CN Planktonic (cells/mg cells)/(cells/mg cells)	93.8
CN Turbulent biofilm/CN Laminar biofilm (cells/mg cells)/(cells/mg cells)	22.8
CPT Planktonic/CPT Turbulent biofilm (pg/cell)/(pg/cell)	703
CPT Planktonic/CPT Laminar biofilm (pg/cell)/(pg/cell)	131
CPT Laminar biofilm/CPT Turbulent biofilm (pg/cell)/(pg/cell)	5.37
CPL Planktonic/CPL Turbulent biofilm (pg/cell)/(pg/cell)	25714
CPL Planktonic/CPL Laminar biofilm (pg/cell)/(pg/cell)	290
CPL Laminar biofilm/CPL Turbulent biofilm (pg/cell)/(pg/cell)	88.7

It can be observed that planktonic cells have a higher RA than biofilms formed either under turbulent and laminar flow, being laminar biofilms less actives than those formed under turbulent flow. The cell numbers found in the equal amount of biological mass (biofilm or bacteria) is much higher in biofilm when comparing with planktonic situation. Comparing biofilms, those formed under turbulent flow have about 23 times more cells per mg of biofilm than laminar biofilms. Concerning cellular biochemical composition (proteins and polysaccharides), planktonic cells have a much higher content than biofilms, being this difference much more pronounced for cells within biofilms formed under turbulent biofilms and for the intracellular polysaccharide content.

Figure 1 shows SEM photomicrographs of biofilms formed under turbulent and laminar flow.



A B

Figure 1. SEM photomicrographs of a 7 d old *Pseudomonas fluorescens* biofilms formed under turbulent (**A**) and laminar (**B**) flow, on stainless steel slides.

Biofilm architecture is controlled by the hydrodynamic conditions as can be seen in Figure1. Biofilms grown under turbulent flow look very differently from those grown in laminar flow, emphasizing that hydrodynamic conditions play an important role in the biofilm architecture.

DISCUSSION

This work shows that biofilm developed in turbulent flow (Figure 1) look and behave (Table 1) very differently from those grown in laminar flow. Cells within biofilms are metabolic and biochemically different from planktonic cells. Several authors (Sauer and Camper, 2001; Sauer *et al.,* 2002) already reported the existence of considerable evidences that indicated that bacterial cells attached to, and growing on, surfaces in biofilms are physiologically distinct from their planktonic grown counterparts. These differences have significant impact when planktonic cells were used as the basis of the design of biofilm control procedures. One of the earliest observations of such altered behaviour is the increased resistance of biofilm cells to antimicrobial agents when comparing planktonic and biofilm states and biofilms formed under different flow regimes (Simões *et al.,* 2003). Parallel studies, performed with the same strain reported in this work have shown that bacterial attachment to SS surfaces under different flow regimes induced changes in the protein expression profiles. Phenotypic changes were also detected as a consequence of biofilm formation (results not shown).

Comparing the effects of the flow regime in the biofilm behaviour, our study is in accordance with the one found by Pereira *et al.* (2002b), that determined the biovolume of biofilms. Those authors stated that, under turbulent conditions the biofilm contained more cells than under laminar conditions, being this fact related with the increased shear stress, rather than with quorum sensing phenomenon (Purevdorj *et al.,* 2001). Pereira *et al.* (2002b) also found that biofilms formed under turbulent flow are thicker than the ones formed under laminar flow. Since thickness can give rise to increased diffusional limitations, that biofilm property have thus obvious influence on the microbial metabolism of the entrapped cells. Nevertheless, biofilms formed under turbulent flow are more actives, being, probably, the glucose consumption due to the higher number of cell per g of biofilm, which determined differences in the biochemical characteristics.

CONCLUSIONS

Significant differences were encountered when comparing planktonic with biofilm cells and with cells within biofilms formed under different flow regimes. Planktonic cells are more active than when they are entrapped within a biofilm. Planktonic cells have also the major amount of intracellular proteins and polysaccharides. This fact may be related with the decrease of the cellular size (results not shown) as a consequence of the hydrodynamic conditions. Turbulent biofilms have

a higher number of cells, having, however, those cells less intracellular protein and polysaccharide content. This more detailed understanding of the complex roles of environmental factors in the biofilm behaviour will lead to improve the strategies for biofilm control.

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