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# The Role of Exopolymers Produced by *Sphingomonas paucimobilis* in Biofilm Formation and Composition

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Exopolymers have been associated with the initial adhesion of bacteria, which is the primary step for biofilm formation. Moreover, the polymeric matrix of biofilms has a considerable influence on some of the most important physical and physiological properties of biofilms. The role of extracellular polymers in biofilm formation was studied using three mutants of *Sphingomonas paucimobilis* with increasing capabilities for exopolymer production. The physical, biochemical and physiological properties of three different layers of each biofilm were determined. The layers were detached by submitting the biofilm to increasing shear stress. The results revealed that the presence of exopolymers in the growth medium was essential for biofilm formation. The mutant producing the highest amount of exopolymer formed very thick biofilms, while the biofilms formed by the medium exopolymer producer were on average 8 times thinner. The lowest exopolymer producer did not form biofilm. In both types of biofilms, exopolymer density increased with depth, although this tendency was more significant in thinner biofilms. Cell distribution was also more heterogeneous in thinner biofilms, exhibiting a greater accumulation of cells in the inner layers. The thicker biofilms had very low activity in the inner layer. This was related to a high accumulation of proteins and DNA in this layer due to cell lysis and hydrolytic activity. Activity in the thin biofilm was constant throughout its depth, suggesting that there was no nutrient limitation. The production of exopolymers by each cell was constant throughout the depth of the biofilms, although it was greater in the case of the higher producer.

*Keywords:* biofilm; *Sphingomonas paucimobilis*; polymeric matrix; exopolysaccharides

## INTRODUCTION

Deposition of microorganisms on surfaces and biofilm formation is an important bacterial survival strategy. Biofilms occur spontaneously on both inert and living systems, being of concern to a wide range of scientific disciplines. In industry, biofilms can have a detrimental impact on account of the undesirable effect of biocorrosion promoted by microbial cell accumulation at interfaces (Characklis & Cooksey, 1983). They can also pose health problems due to the occurrence of diseases related to the colonization of bacteria on tissues and implants (Neu *et al.*, 1992; Wilcox, 1993). On the other hand, biofilms have a beneficial role in many biotechnological processes such as water and waste

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water treatment (Lazarova & Manem, 1995). A thorough understanding of biofilm accumulation, growth and composition is necessary to operate biotechnological processes efficiently and to eliminate biofilms effectively when they cause problems.

Biofilms are formed by microbial cells embedded in an exopolymeric matrix. The extracellular matrix is mainly composed of polysaccharides and proteins, although other compounds such as DNA and humic substances may also be present (Nielsen *et al.*, 1997; Jahn *et al.*, 1999). Exopolymeric substances (EPS) have a role in biofilm formation due to their involvement in bacterial adhesion. EPS also determine many important properties of the biofilm such as strength, elasticity and sorption capacity. Moreover, the exopolymeric matrix constitutes an important food reservoir and has a protective effect against toxic substances such as biocides and antibiotics (Morton *et al.*, 1998).

*Sphingomonas paucimobilis* was chosen for the present study because it is an ubiquitous bacterium in soil, water and sediments. This bacterium has considerable potential in bioremediation and waste water treatment due to its capacity to degrade aromatic compounds (Fredrickson *et al.*, 1995). The strain ATCC 31461 (Kang & Veeder, 1982) is able to produce high amounts of an anionic EPS called gellan which is composed of repeated units of a tetrasaccharide with two molecules of D-glucose, one of D-glucuronic acid and one of L-rhamnose (Pollock, 1993). Moreover, well defined mutants defective in EPS synthesis are available that constitute an important tool to investigate the importance of EPS in biofilm formation (Richau *et al.*, 1997a; 1997b).

The principal aim of this work was to study the influence of exopolymers in biofilm formation and cohesion strength. Analysis of detached layers of the biofilm also enabled the role of thickness in biofilm activity, structure and composition to be evaluated.

## MATERIALS AND METHODS

### Bacterial Strains

The gellan producing strain *Sphingomonas paucimobilis* (ATCC 31461) (formerly *Pseudomonas elodea*) was used after $Cu^{2+}$-stressed cultivation to derive three mutants (TR, CV and F72) that were defective in gellan synthesis (Richau *et al.*, 1997a). TR is a high gellan producer, CV a medium producer and F72 a low gellan producer. The mutant cells were supplied by the Instituto Superior Técnico, Portugal.

### Biofilm Formation

The biofilms were grown in a 500 ml reactor containing four suspended and immersed glass cylinders. The reactor was continuously fed with aerated S medium (containing $l^{-1}$ of distilled water 12.5 g $Na_2HPO_4 \cdot 2H_2O$, 3 g $KH_2PO_4$, 1 g NaCl, 1 g $K_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.001 $FeSO_4 \cdot 7H_2O$, 5 g glucose, 1 g yeast extract (DIFCO) and 1 g casein (Difco)).

A batch culture of each mutant in the exponential growth phase was placed inside the reactor ($R_F$). The culture was grown in batch over 2 h, after which the reactor was fed with medium at increasing dilution rates (0.03 $h^{-1}$, 0.06 $h^{-1}$ and 0.2 $h^{-1}$).

In all experiments, effluent samples were taken periodically for polysaccharide, biomass and glucose quantification.

### Biofilm Characterisation

After 12 d operation, the glass cylinders covered with biofilm were carefully removed from the reactor. Each cylinder was submitted to a set of procedures presented in Figure 1. The wet weight was determined by weighing the cylinder with biofilm before the removal assay. The volume was determined by immersing the cylinder covered with biofilm in a glass container filled with distilled filtered water and the

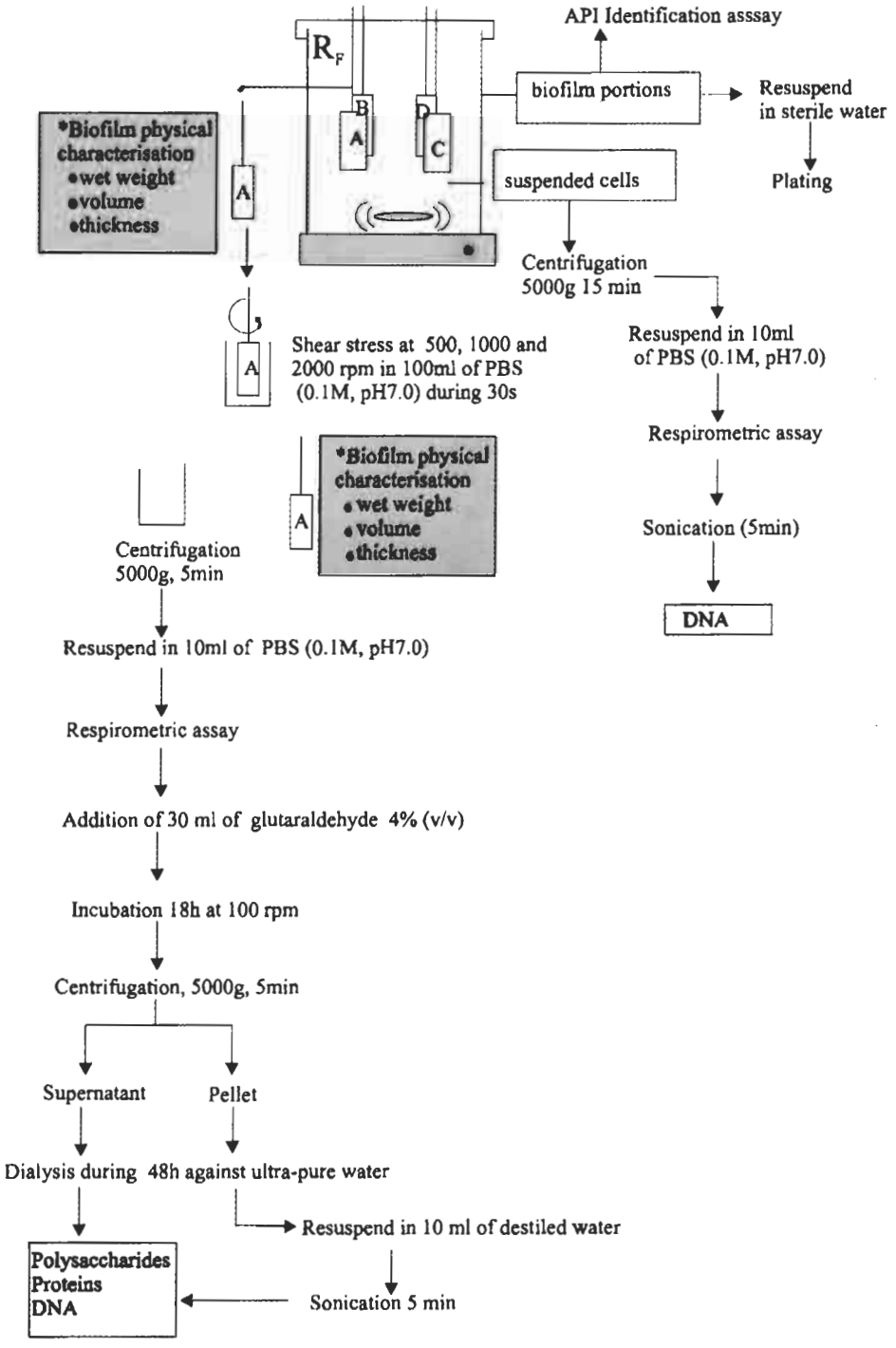


FIGURE 1 Steps involved in the characterisation of biofilm.

overflow water was recovered and weighed. The biofilm thickness was determined with the aid of a video camera having a 100 × magnification lens. The needle of a micrometer was placed on the top of the biofilm, and allowed to penetrate the biofilm until it reached the surface by

rotating the micrometer knob, and the thickness registered.

The phenotypic control of the immobilised biomass was assessed *via* the API-Analytic Profile Index identification test (API 20 NE, Biomérieux). This is a standardised micro-method combining eight conventional and twelve assimilation tests for the identification of non-fastidious Gram-negative rods not belonging to the Enterobacteriaceae (Peladan & Monteil, 1988).

Each detached biofilm portion was submitted to an extraction procedure using glutaraldehyde as described previously (Azeredo *et al.*, 1999a), in order to separate the polymeric matrix from the cells. The extracted solution (corresponding to the polymeric matrix) was recovered by centrifugation (5000 × g, 5 min). To remove the glutaraldehyde, which interferes with the subsequent analytical methods, both pellet and supernatant were dialysed for 48 h, using a cellulose membrane (Medicell) with a molecular weight cut off (MWCO) of 14000. The relative composition of the polymeric matrix was determined by measuring DNA, proteins and polysaccharides (against standards) in the dialysed supernatant. The dialysed pellet was sonicated for 5 min and the DNA content was determined and correlated to the amount of cells using Eqn (1), obtained experimentally with a correlation coefficient of 0.999:

$$\text{Number of cells } l^{-1} = \frac{\text{DNA}(\text{mg } l^{-1}) + 0.2982}{3 \times 10^{-11}} \quad (1)$$

### Biofilm Activity

A Biological Oxygen Monitor (YSI – Model 5300) was used to measure the rate of biofilm specific oxygen uptake, OURspe, $g\,O_2\,cell^{-1}\,min^{-1}$, according to the method described by Domingo *et al.* (1997).

### Analytical Methods

DNA was determined using DAPI, according to Brunk *et al.* (1979). Total protein was quantified by the Lowry modified method, using the protein assay kit SIGMA P5656 with bovine serum albumin (BSA) as standard. Polysaccharides were determined by the phenol-sulphuric acid assay (Dubois *et al.*, 1956), using glucose as standard. Glucose was determined according to the method described by Miller (1959).

The amount of planktonic cells in the reactor was determined in the effluent recovered from the reactor after centrifugation (5000 × g, 15 min). The supernatant containing EPS in solution (that would interfere with the measurements) was discharged. The pellet was resuspended to the initial volume in phosphate buffered saline, pH 7.0 ($0.29\,g\,l^{-1}$ $KH_2PO_4$, $1.19\,g\,l^{-1}$ $K_2HPO_4$, $4.93\,g\,l^{-1}$ NaCl) and its absorbance was determined at 640 nm.

## RESULTS

### Biofilm Formation and Growth

The continuous operation of the reactors started with a low dilution rate ($0.03\,h^{-1}$). In the reactor where the TR biofilm was formed, the concentration of biomass in suspension increased after 72 h operation (Figure 2), whilst in the reactor where the CV biofilm was grown, a slight decrease in the concentration of suspended cells was detected (Figure 3). After this period, the dilution rate was duplicated and the TR cells in suspension decreased, together with the concentration of glucose, suggesting that some substrate had been used by the immobilised biomass. The same was observed with the CV suspended biomass after 6 d operation. Finally, a dilution rate of $0.2\,h^{-1}$ (1.2 times the specific growth rate determined in a previous study) was used and the CV suspended cells were washed out. After 12 d operation, mutant TR, which produced the greatest amount of

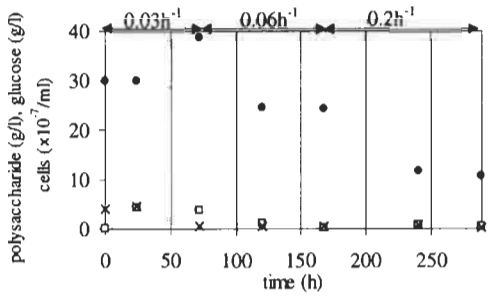


FIGURE 2 Variation in the concentration of glucose (□), polysaccharides ( × ) and cells (•) with time, in the reactor where biofilm TR was formed, under different dilution rates (↔).

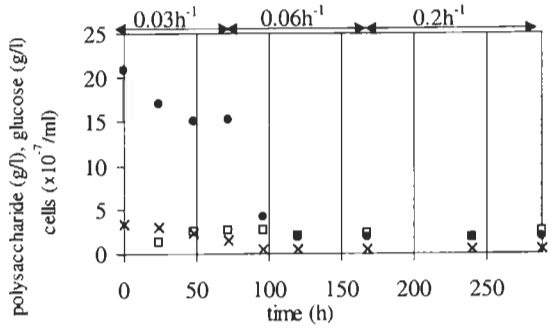


FIGURE 3 Variation in the concentration of glucose (□), polysaccharides ( × ) and cells (•) with time, in the reactor where biofilm CV was formed, under different dilution rates (↔).

exopolymer (5.1 g $l^{-1}$ in batch culture), had formed a very thick biofilm (1.1 mm on average). The biofilms formed by CV, the medium polysaccharide producer (4.3 g $l^{-1}$ in batch culture), were on average 8 times thinner than those produced by TR. The mutant F72, the lowest polysaccharide producer (2.5 g $l^{-1}$ in batch culture) did not form biofilms under these operating conditions. The amount of cells in suspension decreased with increasing dilution rate, although for higher dilution rates some TR cells remained in solution (Figure 2).

## Physical Characteristics of the Biofilm

The physical characteristics of the biofilm formed by TR cells are summarised in Table I. Using a rotation speed of 500 rpm, 47.3% of the total biofilm thickness was removed. This portion had a very high water content (99.6%) and a low density. The second layer removed, using twice that velocity, corresponded to 34.3% of the thickness of the total biofilm. Finally, 11.2% of the biofilm was detached at 2000 rpm, leaving a thin layer of 78 μm, corresponding to 7.2% of the total biofilm thickness. From Table I it can be seen that biofilm density increased from the outer layer (removed at 500 rpm) to the inner layer (removed at 2000 rpm).

The physical characteristics of biofilm formed by CV cells can also be seen in Table I. With a rotation speed of 500 rpm, the greatest portion of biofilm was removed (52% of the thickness of total biofilm). This biofilm was easier to detach than the biofilm produced by TR, indicating that cohesion forces in TR (the highest producer of exopolymer) biofilm are stronger than those in CV biofilm. A rotation speed of 1000 rpm removed only 9% of the thickness of the total biofilm. At 2000 rpm 15% of the biofilm was removed, indicating that the inner biofilm layers were more difficult to detach. Biofilm with a thickness of 33 μm remained on the cylinder wall after detachment at 2000 rpm. Although the

TABLE I Physical properties of TR and CV biofilm layers detached at 500, 1000 and 2000 rpm

| Physical properties | Thickness (μm) | | Wet weight (g) | | Density (mg total solids $cm^{-3}$) | |
|---|---|---|---|---|---|---|
| Rotation speed (rpm) | TR | CV | TR | CV | TR | CV |
| 500 | 512.5 ± 74 | 70.0 ± 9 | 2.48 ± 0.3 | 167.0 ± 22 | 4.82 ± 0.9 | 16.4 ± 3 |
| 1000 | 370.9 ± 57 | 12.0 ± 2 | 1.40 ± 0.2 | 40.0 ± 5 | 8.18 ± 1.7 | – |
| 2000 | 121.0 ± 13 | 21.0 ± 3 | 0.40 ± 0.1 | 60.1 ± 7 | 19.51 ± 3.9 | 77.0 ± 15 |

remaining layer was 2.4 times thinner than the non-detached layer of TR biofilm it corresponded to 24% of total biofilm formed in the cylinder. The density of CV biofilm increased greatly from the outer (removed at 500 rpm) to the inner layer (removed at 2000 rpm).

It should be stressed that discrete biofilm sections were removed uniformly, as verified by the SD of the thickness of each of the remaining portions. Such uniformity suggests that the biofilm layers are held by different cohesion forces.

### Composition of the Biofilm

The removed layers of biofilm were analysed in terms of macromolecular composition and cell number (Figures 4 and 5). The thinner biofilm, formed by the lowest EPS producer (CV), presented a heterogeneous distribution of cells along its depth. In the inner layers (removed at 1000 and 2000 rpm) a great accumulation of cells was found (Figure 5). The amount of immobilised cells in the TR biofilm was lower and their distribution within the layers that were removed was more homogeneous (Figure 4).

Proteins and polysaccharides were the major fraction of the EPS of both biofilms. In the TR biofilm, a greater accumulation of protein and DNA was found in the inner layer (Figure 4). The presence of these two components can be associated with natural cell lysis. However, it is important to ensure that these components were not released from cells as a result of the extraction procedure. The method used in this study was optimised in previous studies (Azeredo *et al.*, 1998; 1999a), in order to extract high amounts of EPS without promoting cell lysis or permeabilisation. The matrix of the outer layer of TR biofilm (removed at 500 rpm) showed a lower amount of protein and DNA per cell than the inner layer (removed at 2000) (Table II). However, the increase in the amount of proteins per cell was greater than that of DNA per cell. The ratio of proteins/polysaccharides obtained in the portion of TR biofilm removed at 500 rpm was 1.6 times smaller than in the biofilm portion removed at 1000 rpm, and 3 times smaller than that detached at 2000 rpm. The increase in the ratio of proteins/polysaccharides is in accordance with the increase in protein content per cell.

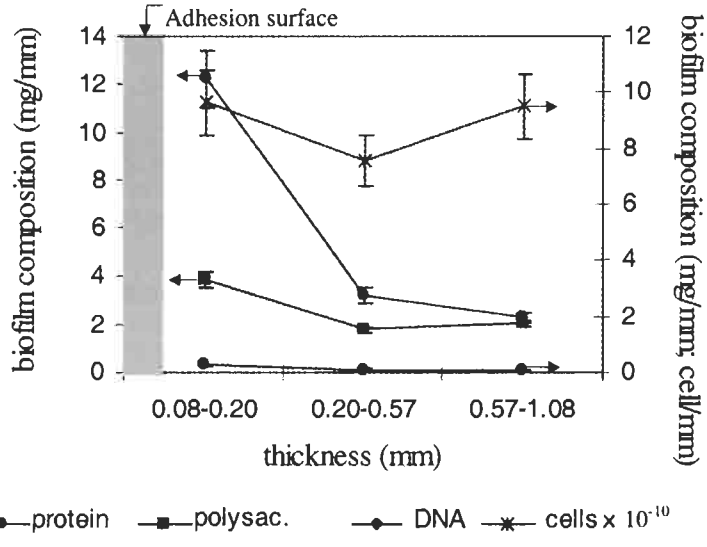


FIGURE 4 Variation in the composition of proteins (—●—), polysaccharides (—■—), DNA ( —◆—), and cells ( —✱—) throughout the depth of biofilm TR.

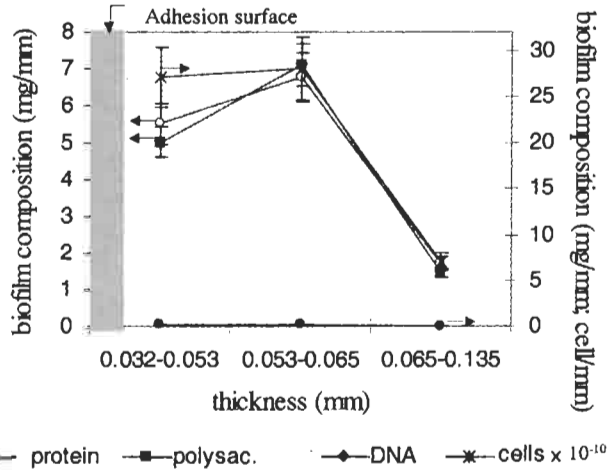


FIGURE 5 Variation of the composition of proteins (—o—), polysaccharides (—■—), DNA (—◆—), and cells (—✱—) throughout the depth of biofilm CV.

TABLE II Composition of TR and CV biofilm matrices per cell removed at 500, 1000 and 2000 rpm

| Composition | Prot/cell ($10^{-14}$ g cell$^{-1}$) | | Pol/cell ($10^{-14}$ g cell$^{-1}$) | | DNA/cell ($10^{-16}$ g cell$^{-1}$) | | Prot/Pol (mg mg$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| Rotation speed (rpm) | TR | CV | TR | CV | TR | CV | TR | CV |
| 500 | 2.39 | 2.40 | 2.11 | 2.09 | 7.14 | 6.76 | 1.13 | 1.15 |
| 1000 | 4.21 | 2.25 | 2.33 | 2.54 | 12.80 | 7.06 | 1.81 | 0.89 |
| 2000 | 13.10 | 1.95 | 3.97 | 1.91 | 25.70 | 5.59 | 3.30 | 1.02 |

Prot = protein; Pol = polysaccharide

The amount of proteins and polysaccharides present in the EPS matrix of CV biofilm followed the same distribution as the cellular biomass (Figure 5). Accordingly, the specific composition of the EPS matrix (protein per cell, polysaccharide per cell and DNA per cell) was constant throughout the depth of this thin biofilm (Table II).

## Biological Properties of the Biofilm

The phenotypic characteristics of immobilised cells were analysed using an API assay and compared with those of cells grown in suspension. The API identification test showed that TR cells from TR biofilm had the ability to degrade gelatine, which is not common in this strain. This property is usually considered to be associated with the production of proteolytic enzymes.

## Biofilm Activity

The oxygen uptake rate (OUR) of entrapped cells in the different biofilms, as well as of cells grown in suspension, was determined by respirometry. This technique can be used to estimate the activity of cells and also to determine their metabolic viability (Huang *et al.*, 1984; Domingo *et al.*, 1997). The specific respiratory activities of TR and CV biofilms were compared with those of TR and CV cells grown in suspension in the reactor (Figures 6 and 7). Figure 6 shows that in the thicker biofilm (produced by TR) activity decreased throughout the depth of the biofilm and was almost zero in

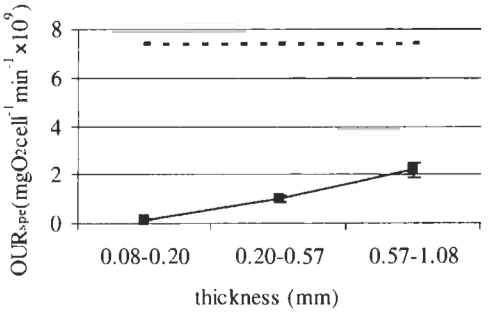


FIGURE 6 Specific oxygen uptake rate (OURspe) of biofilm TR; (■) = biofilm layer; (- - -) = cells in suspension.

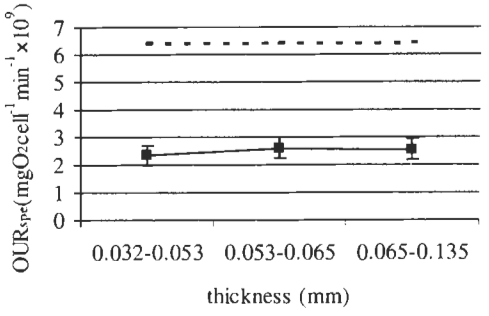


FIGURE 7 Specific oxygen uptake rate (OURspe) of biofilm CV; (■) = biofilm layer; (- - -) = cells in suspension.

the inner layer. In the thinner biofilm (produced by CV), activity was constant throughout the biofilm depth (Figure 7). Thus, this biofilm was probably not nutrient limited. Figures 6 and 7 show that the activity of suspended cells was higher than that of the biofilm portions.

## DISCUSSION

The production of biofilm is normally performed by washing out the biomass immediately after the initial contact between cells and the substratum (Heijnen *et al.*, 1992). In the present case, this procedure was not successful and biofilm formation was only possible by starting the operation with a very low dilution rate. This highlights the importance of exopolymers in biofilm formation. In a previous study it was found that the presence of EPS enhanced the adhesion of TR and CV cells to glass (Azeredo *et al.*, 1999b). Also the presence of exopolymer in solution in the initial stage of biofilm formation (Figures 2 and 3) supports cell–cell adhesion. TR, the highest EPS producer gave rise to thick biofilms (1.1 mm on average) whereas CV, the medium producer formed thin biofilms (0.13 mm on average). It must be noted that the mutants used in this study are able to produce the same exopolymer as the wild strain, although in different amounts and with different degrees of polymerisation (Richau *et al.*, 1997a; 1997b).

At high dilution rates, the wash out of suspended cells only occurred for CV biofilm. In the case of TR biofilm, some cells remained in suspension (Figure 2). It is possible that some TR biofilm would have become detached, resulting in suspended biomass. This phenomenon is very likely to occur in thick biofilms growing in rich media (Characklis & Cooksey, 1983).

Besides being involved in the initial formation of biofilms, EPS are also important in the stability of biofilms; TR biofilm was more difficult to detach than CV biofilm, suggesting that the cohesion forces within a biofilm formed by a high EPS producing strain are higher than those formed by a medium EPS producer.

Analysis of the detached layers (Table I) showed the stratified structure of both biofilms revealed by different strengths of cohesion, biochemical composition and an increase in density within the biofilm. Some authors (Hoehn & Ray, 1973; Zhang & Bishop, 1994) also reported an increase in density along the depth of biofilms. The data summarised in Figure 8 indicate that an increase in biofilm density is related to an increase in cell accumulation. This phenomenon can be explained by the fact that the growth of bacteria in the inner layers results in an increase in density, while the growth of bacteria in the outer layers leads to an increase in the biofilm thickness. The increase in density within CV biofilm was more significant than in

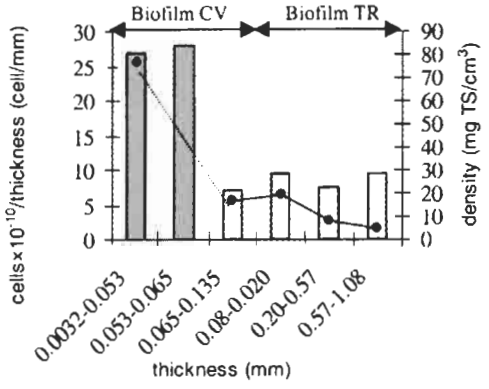


FIGURE 8 Number of cells normalised by biofilm thickness ( ) and biofilm density (•——•) throughout the depth of CV and TR biofilms.

TR biofilm (Figure 8). According to Zhang (1994), biofilms with a thickness $\leq 140\,\mu m$ (which is the case for CV biofilm) are fully penetrated by oxygen whereas biofilms thicker than $130\,\mu m$ are only partly penetrated. This statement, together with experimental evidence, suggest that in CV biofilm (the thin biofilm), the density increases rapidly because there is no lack of nutrients near the adhesion surface. In the thick biofilm (TR) substrate limitation would be imposed at the biofilm base, triggering a decrease in cell growth.

Nutrient limitation is very likely to have occurred in the deepest layers of TR biofilm. From the data shown in Figure 6, it is clear that the inner layer was almost inactive and the metabolic activity decreased strongly with increasing depth of the biofilm. As the detached layers of CV biofilm did not exhibit great differences in cell activity (Figure 7), it can be concluded that this biofilm was not nutrient limited. However, the activity of suspended CV cells is greater than the activity of the cells entrapped in biofilm (Figure 7). The same can be observed for TR biofilm (Figure 6). Some authors have reported that the metabolic activity of adhering bacteria is greater than that of suspended cells when the nutrient concentration is low (Atkinson & Fowler, 1974; Hamilton, 1987). On the other hand, Jeffrey and Paul (1986) concluded that the metabolic activity of adhering cells could decrease when the nutrient concentration is high. In the present case, it is possible that since both biofilms were grown in a very rich medium, the detached cellular biomass was able to grow in suspension and exhibit a high activity.

The extracellular matrix of the two biofilms had different compositions. The amount of DNA per cell measured in the matrix of the inner layer of TR biofilm was greater than that obtained in the deeper portion of CV biofilm. DNA is an important constituent of biofilm matrices, contributing to increased biofilm stability due to its negative charge. The presence of this molecule is commonly associated with natural cell lysis, which normally occurs in old biofilms. As the inner layer of TR biofilm was almost inactive (Figure 6), cell lysis could have occurred, resulting in the accumulation of DNA.

Proteins are also important constituents of biofilms (Jahn *et al.*, 1999). Moreover, *S. paucimobilis* is able to excrete proteins in batch cultures (results not shown). Therefore, the presence of proteins in the polymeric matrix of these biofilms is to be expected. Figure 4 shows a significant increase in the amount of protein in the inner layer of TR biofilm. Moreover, the ratio of protein/cell is very high in this layer (Table II). As already discussed, this may be explained by cell lysis. However, it must be noted that the ratio of protein/DNA also increased in this layer. From the API identification test, the cells entrapped in TR biofilm had the ability to excrete hydrolytic enzymes. Extracellular hydrolytic activity is very common in biofilms, occuring mainly when the biofilm is grown in unfavourable conditions (Jones & Lock, 1989; Sinsabaugh *et al.*, 1991). Thus, cell lysis together with proteolytic activity, as a result of nutrient limitation, are both responsible for the high content of protein in the inner layer of TR biofilm.

The amount of DNA per cell present in the matrix of the inner layers of CV biofilm was

smaller than that obtained from the deeper portion of the TR biofilm (Table II). A similar observation can be drawn for the ratio of proteins/cell content. Moreover, the API identification test did not point to any proteolytic activity in CV biofilm. These facts can be explained by the absence of cell lysis and hydrolytic activity in the inner layer of CV biofilm, probably due to the thinness of this biofilm.

Regarding the polysaccharide content of the biofilm matrix, it has been reported that the production of exopolymers increases under oxygen limitations (Applegate & Bryers, 1991). On the other hand, it was found that these bacteria produce smaller amounts of polysaccharide in batch cultures when the concentration of glucose decreases (results not shown). According to the values presented, the contribution of each cell to the polysaccharide in the matrix of TR biofilm increased slightly in the inner layer (Table II). It is possible that due to the limitation of nutrients (oxygen and glucose) both effects had occurred.

The amount of cellular biomass decreased from the inner to the intermediate layer of TR biofilm. However, it increased in the outer layer. A high cellular density in the outer layer of biofilms has been reported by other authors (Neu & Lawrence, 1997). This can be explained by the absence of nutrient limitation at the surface of biofilm, leading to a fast growth. Cells in CV biofilm were also not nutrient limitated and consequently, accumulation of cellular biomass took place in the inner layers. This behaviour is typical of thin biofilms grown in rich media (Wimpenny & Colasanti, 1997).

In conclusion, exopolymers have a determinant role in *S. paucimobilis* biofilm formation as they are responsible for cell–cell adhesion, thus enhancing the growth of biofilm. Furthermore, they strengthen the cohesive forces within the biofilm, thereby contributing to greater biofilm stability. Biofilm structure and composition appear to be influenced by thickness. Cells entrapped in the thin biofilm (CV cells) were metabolically active, within a relatively homogeneous matrix. Cells in the inner layer, where nutrients were not limited, produced a more heterogeneous, denser biofilm structure. By contrast, the inner layer of the thicker biofilm (TR) was metabolically inactive. Nutrient limitation in the deeper layer of this biofilm was responsible for cell lysis and production of proteolytic enzymes, giving rise to the heterogeneous biochemical composition of the extracellular matrix. However, the cell distribution throughout the depth of this thicker biofilm was more homogeneous.

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