Extraction of exopolymers from biofilms: the protective effect of glutaraldehyde

J. Azeredo, M. Henriques, S. Sillankorva and R. Oliveira

Centro de Engenharia Biológica–IBQF, Universidade do Minho, 4710-057 Braga, Portugal

Abstract The extraction of the exopolymeric matrix is a prerequisite to properly assessing the composition of the biofilm. Several extraction methods have already been developed, however, no universal method has yet been adopted because the compromise between high yields of extraction and minimum cell lysis is difficult to establish. In fact, most of the extraction methods promote leakage of intracellular material. The most common extraction methods, Dowex resin and sonication, were assayed in biofilms of *Pseudomonas fluorescens* and *Alcaligenes denitrificans* submitted to a pre-treatment with glutaraldehyde (GTA). The assessment of ATP released after extraction was used as a criterion of cell lysis. The results showed that GTA is a protective agent against cell lysis. The pre-treatment with GTA is particularly useful combined with sonication.
Keywords ATP; biofilm; cell lysis; extraction methods; glutaraldehyde

Introduction

To estimate the accurate number of cells present in a biofilm and to characterize the polymeric matrix, an extraction is required to separate microbial cells from the exopolymeric substances. There is no universal extraction method, although the methods reported have merit depending on the intended use of the product. Nevertheless, the separation of the exopolymeric matrix without destruction of cells is an important prerequisite of any useful extraction method in order to avoid contamination of extracellular material with intracellular substances. In a previous study the most common extraction methods: Dowex and sonication were compared with the method that uses glutaraldehyde (GTA) and the results showed that GTA was able to extract high amounts of exopolymers with a minimal cell lysis. In fact, GTA seemed to exhibit a protective effect on cells against lysis (Azeredo *et al*., 1999). Based on this assumption, the objective of this work is to test the protective effect of GTA in the extraction by sonication and Dowex resin.

Material and methods

Samples

The extraction methods were assayed in *Pseudomonas fluorescens* biofilms formed under the conditions described by Pereira *et al*. (2000) and *Alcaligenes denitrificans* biofilms produced as described by Teixeira and Oliveira (2000). These methods were also tested in planktonic cells of *P. fluorescens*. For this purpose, a continuous pure culture of the *P. fluorescens* was grown in a 2 L glass fermenter, at 27 ± 1°C, suitably aerated and magnetically agitated. The fermenter was continuously fed with 0.05 L/h of a sterile nutrient solution consisting of 5 g glucose L^{-1}, 2.5 g peptone L^{-1}, 1.25 g yeast extract L^{-1} in phosphate buffer at pH 7. A suitable amount of *Pseudomonas fluorescens* culture was removed from the fermenter, centrifuged (5,000 rpm, 5 min, 4°C) and washed twice with sterile phosphate buffer (pH 6.0; 0.05 M), to be used in the experiments with cell suspensions.

Pre-treatment procedure

Prior to the extraction procedure, a set of samples (cells and biofilm) were pre-treated with GTA and another set with phosphate buffer (pH 7.0, 0.01 M) used as control, as described

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below. Portions of biofilm or cells (0.5 g of wet weight) were incubated at 4°C for 3 h 30 min with 50 ml of GTA (1.8% w/v) or phosphate buffer. After this period, the samples were centrifuged at 9,000 g for 10 min, and were resuspended in phosphate buffer and centrifuged again. The final pellet was resuspended again in phosphate buffer. The amount of GTA used in the pre-treatment was optimised by performing an adsorption isotherm to microbial cells. Accordingly, the concentration of GTA required to completely saturate 50 ml of a suspension of 0.5 g (wet weight) of cells during 3 h 30 min was 1.8% (w/v).

Extraction methods

Extraction by sonication. The suspensions were sonicated for 30 s, 1 min and 2 min with a 13 mm probe (sonicator, Vibra Cell, Sonics & Materials Inc.), immersed 40 mm in the liquid, using a power output of 36 W. The tubes containing the samples were kept in crushed ice during sonication.

Extraction using Dowex resin. Prior to extraction the Dowex resin (50×8, Na^+ form, 20–50 mesh, Aldrich-Fluka 44445) was washed with the extraction buffer (2 mM Na_3PO_4; 4 mM NaH_2PO_4; 9 mM NaCl and 1 mM KCl, pH 7.0). 50 g of washed Dowex resin per g of volatile solids were added to the suspensions and stirred with a paddle at 600 and 1,000 rpm during 2 and 4 hours.

The extracted portions of the biofilms were collected in the aqueous solution after centrifugation at 9,000 g for 20 min.

ATP measurement

100 µl of a 25-fold dilution of a mixture of luciferin and luciferase (Sigma FL-AAM) were added to100 µl of the extracted solutions. The light transmission was measured in a bioluminometer (Lumac, Biocounter M 25000).

Protein measurement

The total protein content was determined by the Lowry modified method, using the protein assay kit SIGMA P5656 with a standard of BSA (bovine serum albumin).

Results and discussion

The separation of the exopolymeric matrix without cell destruction is an important prerequisite of an extraction procedure. Different methods have been employed to assess the extent of cell lysis. In many studies the accumulation of protein and DNA in the extracted solutions have been taken as an indication of lysis. However, the exopolymeric matrix usually contains large amounts of proteins and DNA (Gehr and Henry, 1983; Frølund *et al*., 1996) making these determinations ineffective. Intracellular enzymes like glucose-6-phosphate dehydrogenase have been used (Frølund *et al*., 1996), however this enzyme is specific for cells that have the glycolitic pathway, which inhibits this assessment to certain kinds of biofilms (e.g. autotrophic).

In this study, ATP was used as an indicator of cell lysis because this molecule is very unstable outside the cell. Moreover, it can also give information about membrane permeabilisation because it can easily leak through pores induced in the membrane on account of its small size (Chung *et al*., 2000).

Figure 1 shows the amount of ATP extracted after different periods of sonication. The extracted ATP increases when increasing the sonication period. However, in the case of biofilms pre-treated with GTA only small amounts of ATP are extracted and this process is time independent. This data clearly shows that GTA has a protective effect against cell lysis.

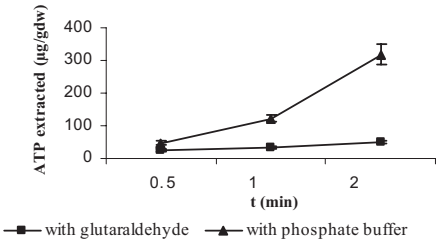

Figure 1 ATP extracted by sonication at 36 W from *P. fluorescens* biofilms with prior treatment with phosphate buffer and glutaraldehyde

Concerning Dowex extraction (Figure 2), very small amounts of ATP are obtained even after 4 h of extraction. Jahn and Nielsen (1995) reported no significant cell-lysis for up to 2 h of Dowex extraction. In fact, the amount of ATP extracted using the Dowex resin can be considered equal to that obtained with sonication after pre-treatment with GTA. It should be stressed that the ATP measurement is very dependent on the type of solution where it is present. In phosphate buffer the amount of ATP is underestimated, while in the presence of glutaraldehyde it is overestimated. To overcome this limitation a standard curve was used for the phosphate buffer. For the ATP measurements in the presence of GTA, the standard curve was elaborated with an average GTA concentration, taking into account that the amount of GTA released is time dependent during the extraction with Dowex resin. This means that for the longer time of extraction a slight overestimation of ATP could still occur.

Biofilms formed by *Alcaligenes denitrificans* under anoxic conditions were also submitted to the extraction procedures. Once more, biofilms pre-treated with GTA released less amounts of ATP after sonication (Figure 3). Concerning Dowex resin, small amounts of ATP are also obtained by this method (Figure 4).

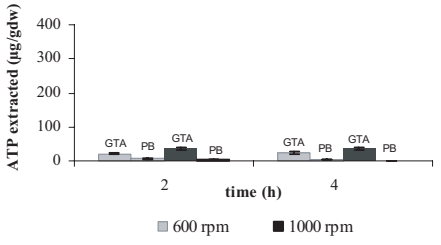

Figure 2 ATP extracted by Dowex resin at 600 and 1000 rpm from *P. fluorescens* biofilms with prior treatment with phosphate buffer (PB) and GTA

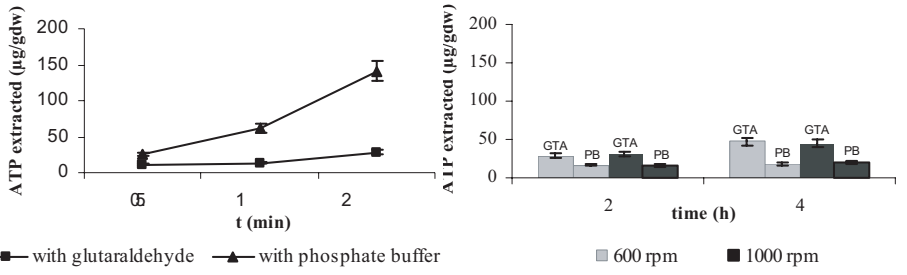

Figure 3 Amount of ATP extracted by sonication at 36 W from *Alcaligenes denitrificans* biofilm with prior treatment with phosphate buffer and glutaraldehyde

Figure 4 Amount of ATP extracted by Dowex resin at 600 and 1,000 rpm from *Alcaligenes denitrificans* biofilm with prior treatment with phosphate buffer (PB) and GTA

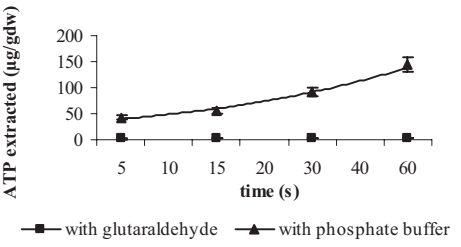

Figure 5 Amount of ATP released by sonication at 36 W from cells of *Pseudomonas fluorescens* with prior treatment with phosphate buffer and glutaraldehyde

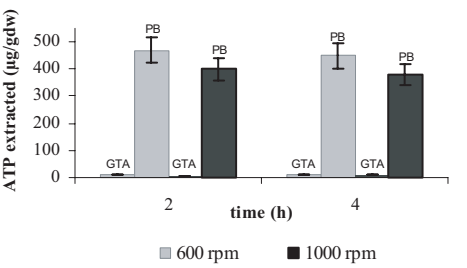

Figure 6 Amount of ATP released by Dowex resin at 600 and 1,000 rpm (B) from cells of *Pseudomonas fluorescens* with prior treatment with phosphate buffer (PB) and GTA

To be sure about the protective effect of glutaraldehyde, cells of *Pseudomonas fluorescens* were submitted to the same extraction procedures. Figures 5 and 6 show the amount of ATP released after sonication and Dowex resin treatment, respectively. For both methods, cells pre-treated with GTA originate much less ATP. These results highlight the protective effect of GTA against the mechanical shear imposed by these two methods.

It is curious to notice that Dowex extraction applied to planktonic cells promoted more cell lysis than sonication, while the opposite occurred in biofilms, which in the present case are very thick (>>1 mm). A possible explanation for this fact lies on the protective effect of the exopolymeric matrix against the mechanical shear stress imposed by this method. So, it is expected that biofilms with a small exopolymeric matrix are more vulnerable to Dowex extraction.

Table 1 presents the amount of protein extracted with Dowex resin and sonication. These results show the protective effect of glutaraldehyde, with which the amount of proteins extracted by sonication is considerable smaller. It must be stressed that special care must be taken in the interpretation of these results because the extracelullar polymers are also comprised of proteins.

Regarding Dowex extraction, as this is a very smooth method, already proved by ATP extraction, it would be expected to extract smaller amounts of protein compared to those obtained by sonication, which is confirmed by the results obtained.

Since glutaraldehyde is frequently used to crosslink proteins it could be possible that the proteins present in the biofilm matrix are crosslinked resulting in a diminishing of the extraction efficiency. If this happens it must be considered as an important drawback of this methodology. The results present in Table 1 suggest that this phenomenon is not likely to occur with the biofilms tested, once, with Dowex extraction, almost the same amount of proteins is recovered applying both pre-treatments.

Table 1 Amount of protein extracted (mg/g dw) by Dowex resin and by sonication from *Alcaligenes denitrificans* and *Pseudomonas fluorescens* biofilms with prior treatment with phosphate buffer (PB) and glutaraldehyde (GTA)

		A. denitrificans biofilm		*P. fluorescens biofilm*	
		GTA	PB	GTA	PB
Sonication	30 s	17.9 ± 1.1	26.1 ± 1.9	55.6 ± 2.9	126.1 ± 9.2
	60 s	19.7± 1.9	35.1 ± 2.2	69.4 ± 3.2	128.3 ± 10.1
Dowex resin (600 rpm, 4 h)		6.3 ± 1.1	7.0 ± 0.9	16.4 ± 1.3	16.5 ± 0.9

Conclusions

The extraction methods used in this study, comprising Dowex extraction and sonication, have been thoroughly described by many authors. Sonication is a very aggressive method leading to the extraction of large amounts of intracellular material (Azeredo *et al.*, 1998, 1999). However, Dowex resin is a much smoother method (Jahn and Nielsen, 1995, 1998). Dowex resin can lead to cell leakage in thin biofilms where the cells are not so protected by the exopolymeric matrix. In the case of sonication, the pre-treatment with GTA is particularly important, because this method is very effective and is not time consuming.

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