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Role of Exopolymeric Substances (EPS) in the Stability of the Biofilm of *Thiomonas arsenivorans* Grown on a Porous Mineral Support

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Biochemical methods were selected to evaluate the role of exopolymeric substances in the stability of biofilms used in bioremediation processes. Biofilms of *Thiomonas arsenivorans* formed on pozzolana were thus treated with pronase (protein target), lectins (Con A or PNA), calcofluor or periodic acid (polysaccharides target), DNase (DNA target), and lipase (triglycerides target). Neither protease nor DNase treatments had any effect on bacterial adhesion. Lectins and calcofluor treatments mainly affected young biofilms. Lipase treatment had a noticeable effect on biofilm stability whatever the biofilm age. Results suggest that it would be an increased resistance of mature biofilms that protects them from external attacks.

Keywords: Exopolymeric substances (EPS), pozzolana, biofilm stability, enzymatic and chemical treatments, *Thiomonas arsenivorans*

Exopolymeric substances (EPS) are one of the main components of biofilms. EPS are composed of sugars, proteins, lipids, DNA, vesicles, and cellular fragments, whose composition and concentration vary as a function of organisms and environmental conditions. [18]. EPS were shown to play several roles in the life of biofilms that can be listed as constructive, informative, sorptive, (redox)-active, surface active, and nutritive functions [8].

Biofilms can have biotechnological applications. One of them is the use of bacterial biofilms with bioremediation properties to develop bioprocesses of contaminated waters treatment. For example, the biofilm formed by the autotrophic bacterium *Thiomonas arsenivorans* (*T. arsenivorans*, DSM 16361) is able to oxidize As(III) (toxic and soluble) to As(V) (less toxic and less soluble), and thus is a good candidate for the development of bioreactors for the treatment of As(III)-polluted waters [3]. The development of fixed-bed bioreactors using biofilms of *T. arsenivorans* is under progress [2, 4]. It appears that the improvement of this bioprocess needs a better knowledge on the development and properties of the biofilm [6, 12]. In this bioprocess, the growth support chosen for biofilm development is pozzolana, as it is inert, cheap, and porous (thus allowing a high surface specificity). Recent works showed that studying biofilms grown on pozzolana was not so easy compared with studying biofilms grown on nonporous and flat surfaces [6]. As an example, the porosity of pozzolana makes it difficult to detach bacteria for further counting and biomass quantification, and to stain the biofilm (for bacteria or EPS) for microscopic observations. As the production of EPS (concentration, composition, and characteristics) is influenced by the presence of a growth support (here pozzolana), studying EPS produced by planktonic cells does not give any information on EPS produced by biofilms. Moreover, as each EPS "family" can play several functions [8], the analysis of EPS does not allow predicting the role of EPS in biofilms. All of this explains in part that studies on biofilms are mainly done in microplates or glass chamber slides [9]. Working with biofilms grown on porous and mineral supports like pozzolana thus needs to adapt or develop protocols that are different from those for biofilms grown on supports like polystyrene microplates [6].

The objective of this work was thus to propose an "indirect approach" that allows to study the involvement of EPS in the stability of biofilms grown on a porous growth support. Several enzymatic and chemical treatments were tested (Table 1). These treatments are already used to (i) stain EPS for microscopic observations [10, 16], (ii) quantify EPS, (iii) characterize the chemical properties of EPS [17], (iv) study the role of EPS in the first step of adhesion of bacteria to a growth support, (v) degrade the EPS structure to disperse biofilm [9, 10], (vi) recover microorganisms [5], or (vii) enhance the efficiency of some bioprocesses such as anaerobic digestion in sludge

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Table 1. Chemical and enzymatic treatments used in this study, and targeted EPS.

Treatment	Product reference	EPS targeted	Preferential cleavage specificity	Concentration used in this study
Pronase	Pronase from *Streptomyces griseus* (Ref. 81748, Sigma-Aldrich)	Proteins	Peptide bond	1 mg/ml
Lectin Con A	Lectin-fluorescein isiothiocyanate conjugate from *Canavalia ensiformis* (Ref. 61761, Sigma-Aldrich)	Carbohydrates	Terminal α-D-mannose or glucose [10]	50 µg/ml
Lectin PNA	Lectin from *Arachis hypogaea* (peanut) FITC conjugates (Ref. L7381, Sigma-Aldrich).	Carbohydrates	β-Galactose(1-3)*N*-acetylgalactosamine [10]	50 µg/ml
Calcofluor	Fluorescent brightener 28 (calcofluor white M2R) (Ref. F3543, Sigma-Aldrich)	Carbohydrates	(1→3)-β- and (1→4)- β-D-glucopyranosyl [13]	80 µg/ml
Periodic acid	HIO_4 (Ref. P7875, Sigma-Aldrich)	Carbohydrates	Glycol	0.2 M
DNase (+ 4.2 mM Mg^{2+})	Deoxyribonuclease 1 from bovine pancreas (Ref. D4263, Sigma-Aldrich)	DNA	Nucleic bond	20 µg/ml
Lipase	Lipase from *Chromobacterium viscosum* (Ref. L0763, Sigma-Aldrich)	Lipids	Ester linkages	40 µg/ml

[14, 17]. Thus, enzymes such as pronase are used to target exoproteins [7, 13], lectins [7, 10, 15], calcofluor [13, 19], and periodic acid [13] for exopolysaccharides; DNase for e-DNA [1]; and biosurfactants [9] or lipases [5] for lipids. Here, these treatments were adapted to reveal the role of EPS in the stability of biofilms already formed on pozzolana.

The chemical and enzymatic treatments selected for this study are listed in Table 1. The principle is based on the fact that if EPS are involved in the stability of the biofilm, the treatments, by disturbing the chemical properties and molecular integrity of EPS, will also disturb the stability and integrity of biofilms, and this will have as a consequence the detachment of bacteria. The more bacteria are detached from the biofilm, the more the targeted EPS should play a crucial role in the attachment of bacteria to the biofilm and to the growth support.

It was first necessary to evaluate the toxicity of the treatments on *T. arsenivorans* to be sure that the observed effects on bacteria detachment were only due to perturbations of the matrix and not to bacteria mortality. Some bacteria have already been in contact with some of the treatments [1, 7, 13]. However, the toxicity of each treatment potentially varies from one bacterial strain to the other, and protocols like that developed in this study have not been tested. It is thus necessary to make a toxicity test for *T. arsenivorans*.

Experiments for toxicity evaluation were done using planktonic cells, as they are known to be more sensitive to treatments than sessile ones. Results with planktonic cells will thus be more representative of the potential toxicity of each treatment on *T. arsenivorans*. Planktonic *T. arsenivorans* cells were thus grown in liquid CSM medium for 48 h at 25°C [3]. Bacteria were then incubated with each treatment separately (see Table 1 for concentrations and Table 2 for time incubation). Concentrations of chemicals and enzymes were that usually used in studies on EPS (see references in Table 1). Previous experiments showed that time incubations longer than 30 min decreased the viability of *T. arsenivorans* for most treatments (data not shown). After the treatment was applied, bacterial viability was checked with the LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes, Kit L-13152), using a fluorescence microscope (Zeiss Axio Imager Z1). The percentage of living bacteria

Table 2. Toxicity test: influence of chemical and enzymatic treatments on the viability of *T. arsenivorans* cells.

Treatment	Incubation time	Living bacteria after treatment (%)
No	30 min and 6 h	95.9
Pronase	30 min	96.5
Lectin Con A	30 min	98
Lectin PNA	30 min	97.2
Calcofluor	6 h	98.7
DNase (+4.2 mM Mg^{2+})	30 min	96.4
Lipase	30 min	98
Periodic acid	30 min	0.8

Bacterial viability was determined using the LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes, Kit L-13152).

Table 3. Effects of the various treatments on the stability of biofilms formed by *Thiomonas arsenivorans* on pozzolana as a function of the biofilm age.

Treatment	Treatment efficiency factor		
	4-day-old biofilm	6-day-old biofilm	8-day-old biofilm
Pronase	0	0	0
Lectin Con A	0.42±0.3	0	0
Lectin PNA	0.63±0.12	0	0
Calcofluor	0.46±0.06	0.37±0.24	0.38±0.14
DNase (+4.2 mM Mg^{2+})	0	0	0
Lipase	0.63±0.06	0.31±0.15	0.5±0.26

The treatment efficiency factor was calculated according to the equation (B–A)/A, with A being the amount of bacteria detached from the biofilm in the absence of treatment (control), and B the amount of bacteria detached after the treatment. Experiments were done in duplicate.

was determined and compared with the percentage of living bacteria in a bacterial aliquot not submitted to treatment (Table 2). Results showed that only the treatment with periodic acid was toxic for *T. arsenivorans*, probably because it caused a rapid decrease of pH medium. All the other treatments did not affect cell viability and can thus be used to study the biofilm of *T. arsenivorans* formed on pozzolana.

The effect of the various treatments on the stability of the biofilm of *T. arsenivorans* was then tested (Table 3). Experiments were done on biofilms grown on pozzolana for 4 to 8 days in fed-batch conditions (replacement of the growth medium by fresh medium every 2 days). *T. arsenivorans* was thus grown as biofilm on pozzolana on the CSM medium containing 100 mg/l As(III) without yeast extract in order to avoid biomolecules of yeast extract from "contaminating" the bacterial-produced EPS [12]. After growth, the biofilms were washed with deionized water to eliminate poorly attached bacteria, and then submitted to the different treatments. The amount of detached bacteria after treatment was evaluated by cell counting (Thoma counting chamber, optical microscopy, and magnification 400 ×).

Results showed that treatments with pronase or DNase did not have any effect on the biofilm, whatever the age of the biofilm, as no bacteria were detached from the biofilm. This suggests that EPS as proteins or DNA would not be significantly involved in the adhesion of *T. arsenivorans*. On the contrary, treatments targeting EPS as exopolysaccharides and lipids significantly affected the stability of the biofilm and led to the detachment of a part of bacteria. The involvement of exopolysaccharides is not surprising, as they were previously identified as the main EPS produced by *T. arsenivorans* [12]. Whereas treatment with lipase had an effect on biofilms of 4 days and more, treatments targeting exopolysaccharides had mainly an effect on "young" biofilms of 4 days. At least three hypotheses could explain this result: (i) there is a decrease of exopolysaccharides production when the biofilm becomes older; (ii) exopolysaccharides are still produced but their composition changes, and they are no longer recognized by treatments such as lectins, (iii) with age, the biofilm structure becomes more compact and thus resistant to some treatments. Staining a "mature" biofilm of 11 days with labeled lectins and DAPI, followed by observation using a fluorescent microscope (Zeiss Axio Imager Z1), showed that the biofilm was labeled by lectins, suggesting that exopolysaccharides were still produced and recognized by lectins (data not shown). This strongly suggests that it is a modification of the biofilm structure that becomes more resistant to treatment and would explain the results obtained in Table 3 (third hypothesis).

In conclusion, this work proposes a set of methods to study the role of each type of EPS in bacterial adhesion and biofilm stability, and shows that enzymatic and chemical treatments allow this investigation. This kind of approach is adapted to the study of biofilms grown on porous growth support like pozzolana, and it gives information on EPS production and role without extraction, purification, and chemical analysis of EPS, which are fastidious steps [15]. This approach is based on the fact that selected treatments are specific of the various families of exopolymeric biomolecules. Lectins are known to specifically target sugars, and are widely used for the visualization or characterization of exopolysaccharides in biofilms [16, 17]. However, experiments done by Johnsen *et al*. [10] suggested that the binding of lectins to biofilms could not necessarily prove the presence of specific target sugars in the extracellular polymeric substances in biofilms. The lectins may bind to non-EPS targets or adhere nonspecifically to components of the biofilm matrix. It is thus necessary to use several treatments targeting the same EPS to validate the results, mainly when working with chemical treatments (such as treatments with lectins), as enzymatic treatments are believed to be specific. As shown in this work, treatment with lectins can be coupled to other chemical treatments such as periodic acid or calcofluor.

Our results also demonstrated that the approach developed in this study is a part of a bigger one and has to be coupled to other experiments such as EPS quantification, staining, and microscopy observations, to better understand the role of EPS in the stability of biofilms.

Concerning the biofilm of *T. arsenivorans* grown on pozzolana and its use in fixed-bed bioreactors for bioremediation purposes, this work showed that sugars and lipids are the main EPS involved in bacterial adhesion, and it suggested that the stability of biofilm increased while the biofilm became older. The better stability of the biofilm with age is a significant advantage for the development of fixed-bed bioreactors, mainly because biofilms in treatment bioreactors are submitted to high flow creating

mechanical forces that can detach biofilms. This kind of approach thus gives some information on the "physical (is the biofilm stable and resistant to chemical, physical, or biological attacks?) and chemical (which EPS are mainly produced?) properties" of biofilms. In the case of the development of fixed-bed bioprocesses, such information, taken together with others (bioremediation activity, biomass, microbial diversity of the biofilm, *etc*.), is useful to better characterize the biofilm and its properties, and thus optimize bioprocesses efficiency.

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