Chapter 13

Exopolysaccharides of the Biofilm Matrix: A Complex Biophysical World

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/51213

1. Introduction

Microbial biofilm development is observed on virtually all submerged surfaces in natural and industrial environments. Biofilms are also observed at interfaces as pellicles, or in the bulk of aquatic environments as flocs or granules [1, 2]. A biofilm is a complex structure made of aggregates of microbial cells within a matrix of extracellular polymeric substances (EPS) (Figure 1). The matrix structure constitutes the elastic part of the biofilm. Interstitial voids and channels separating the microcolonies contain a liquid phase, mainly constituted by water. This liquid phase is the viscous part of the biofilm. The EPS matrix provides the biofilm with mechanical stability through these viscoelastic properties [3].

All major classes of macromolecule, i.e., polysaccharides, proteins, nucleic acids, peptidoglycan, and lipids can be present in a biofilm. Although extracellular polysaccharides are considered as the major structural components of the biofilm matrix, extracellular DNA plays an important role in the establishment of biofilm structure [4]. Moreover, nucleases can be regulators of biofilm formation [5]. To get a better understanding of the role of extracellular polysaccharides in the biofilm architecture and mechanical properties, it is necessary to take a look at the properties of a limited number of components, which can be isolated. Most microbial exopolysaccharides are highly soluble in water or dilute salt solutions, and capsule-forming polysaccharides are attached to the cells surface through covalent bonds to other surface polymers. Many of the extracellular polysaccharides produced in biofilms are insoluble and not easily separated from the cells, complicating the precise determination of their chemical structures and physical properties. Jahn et al. extracted a mixture of polymers from *Pseudomonas putida* biofilm material and found it to be very heterogeneous [6]. Most bacterial exopolysaccharides can exist either in ordered or disordered forms. Elevated temperatures and extremely low ionic concentrations favour the disordered forms. Polysaccharide molecules can interact with themselves or with

heterologous ions and molecules to yield gels, often with multivalent cations playing a significant role in the process. Polysaccharides also interact with proteins molecules both as solutes and when attached to the surface of the microbial cells. The polysaccharide - protein interactions in the matrix induce both structural and functional properties. Indeed, some of these proteins are enzymes constituting an external digestion system [7].

Biofilms in differing environments can be exposed to a very wide range of hydrodynamic conditions, which greatly affect the matrix and the biofilm structure [8]. The shear rate determines the rate of erosion of cells and regions of the matrix from the biofilm. Polysaccharides of the matrix exhibit flow and elastic recovery; because of the flexibility of the matrix its shape can change in response to an applied force. The shear stress to which a biofilm is exposed also affects the physical morphology and dynamic behaviour. Biofilms grown under higher shear are more strongly adhered and have a stronger EPS matrix than those grown under lower shear [9]. Biofilm density can be influenced by the fluid shear during growth [10]. *Pseudomonas* biofilms grown under laminar flow generally consist of hemispherical mound-shaped microcolonies, which form an isotropic pattern on the surface [9]. The biofilm microcolonies grown in turbulent flow are elongated in the downstream direction to form filamentous streamers. The streamers are attached to the glass substratum by an upstream head while the downstream tails are free to oscillate in the flow. Thus, hydrodynamics conditions influence both the structure and the material properties of biofilms [9]. This may be related to the physical arrangement of individual polymer strands in the biofilm EPS matrix [11]. The constitution of the biofilm matrix of *S. enteritidis* varies with pressure forces applied to the biofilm. Indeed in the absence of pressure, the sugars in the biofilm matrix are mainly composed of glucose and very little fucose. However in the presence of power flow, the share of fucose in the biofilm matrix is increased from 11% to about 30% [12].

In this chapter, after the presentation of exopolysaccharides extraction and purification from the biofilm matrix, the structural and physical properties of bacterial alginates, cellulose and other exopoysaccharides related to biofilm formation are discussed. An illustration of the complexity of the biofilm matrix architecture and the role of exopolysaccharides in the properties of the matrix is given through biofilms formation at the surface of nanofiltration membranes used for drinking water production.

2. Exopolysaccharides extraction and purification from the biofilm matrix

This section focuses on specific extraction methods targeting exopolysaccharides. General extraction methods for exopolysaccharides are first presented, followed by a presentation of the corresponding exopolysaccharides properties and carbohydrate contents.

2.1. Methods for exopolysaccharides extraction

Exopolysaccharides constitute the main EPS in many biofilms. They form the backbone of a network where other EPS components can be included. The stability of the biofilm matrix is

dominated by entanglement of EPS and weak physicochemical interactions between molecules. These interactions correspond to various binding forces such as electrostatic attractive forces, repulsive forces (preventing collapsing), hydrogen bonds, van der Waals interactions and ionic attractive forces [13].

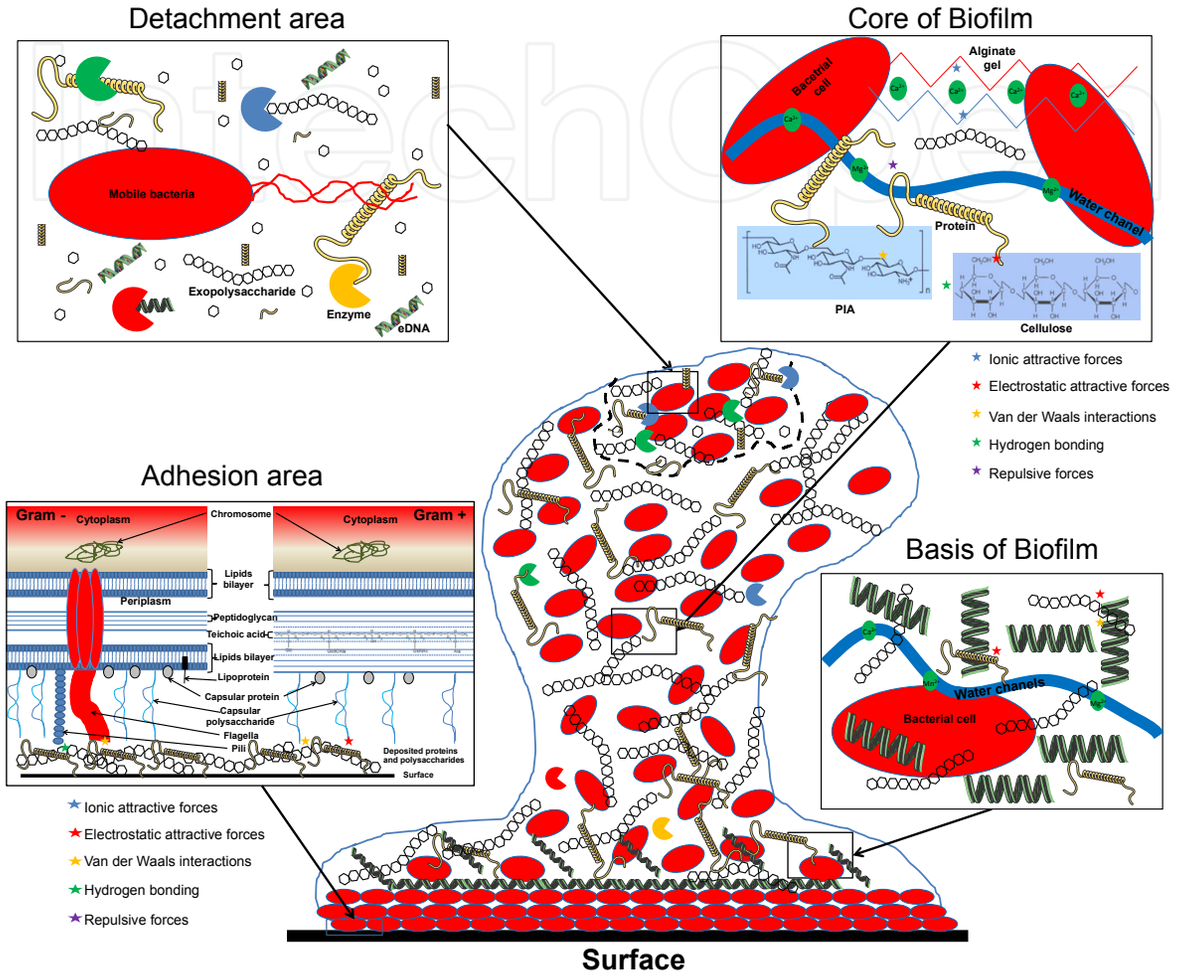

Figure 1. Schematic representation of a mature biofilm. In the centre, overall diagram of the structure of a biofilm to an interface solid / liquid: bacteria are attached to the solid surface and included in a self-induced polymer matrix. In the area of contact between bacteria and surface, the microbial cells can interact with the surface via several protein and polysaccharide appendages (pili, flagella, LPS, capsular polysaccharides) depending on the type of bacteria. On the basis of the biofilm, bacterial cells are embedded in a matrix containing high eDNA concentrations, in addition to proteins and polysaccharides. The eDNA plays a major role in early biofilm formation. In the core of the biofilm, channels of water carrying ions and nutrients cross the biofilm matrix containing high concentrations of exopolymeric substances. All these exocellular compounds form a protective gel around the microorganisms. In the biofilm detachment area, microbial enzymes destroy the exopolymeric matrix and release the cells that regain mobility, to be able to colonize new surfaces.

The exopolysaccharides recovery from the biofilm matrix in order to get a better understanding of their nature, requires to break down the interactions between EPS and selectivity separate them from other EPS and from matrix cells without cell lysis. The

evaluation of cell lysis can be performed by measuring activity of the intracellular marker enzyme glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49). Thus, substantial cell lysis occurring during the EPS extraction is commonly observed [14]. Regarding extraction methods, publications dealing with selective extraction of exopolysaccharides are missing, as already reviewed by Denkaus *et al.* [15]. Indeed, procedures described in the literature mainly deals with EPS extraction.

Physical and/or chemical methods are used to extract EPS from biofilms. Some EPS are tightly associated to the biofilm structure, sometimes through covalent bounds to the cells surface and are not directly extracted. Others free EPS are directly released. The easily released EPS can be separated using physical methods such as high-speed centrifugation and ultrasonication. Indeed, centrifugation is often used to separate soluble EPS from bacterial cells from pure cultures. Firmly cells-associated EPS require chemical methods of extractions. EPS cross-linked by divalent cations can be released from the biofilm matrix by complexing agents such as ethylenediamine tetraacetic acid (EDTA), by cation-exchange resins such as Dowex or by a formaldehyde treatment with or without sodium hydroxide [14, 16].

Various methods used to extract EPS can be applied to the extraction of exopolysaccharides as illustrated on Figure 2.

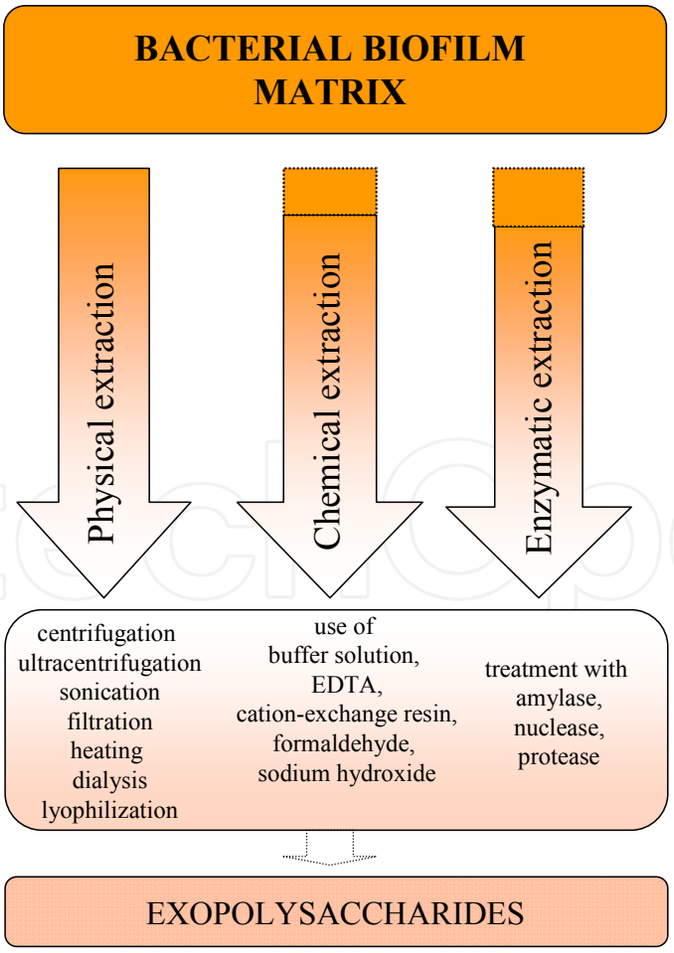

Figure 2. Pathways of exopolysaccharides extraction methods from biofilms

EPS extraction can be done from pure cultures of from complex microbial communities. For example, EPS material can be removed from *Pseudomonas aeruginosa* bacteria by centrifugation at 40 000g for 2 hours at 10°C. Purification of alginate is mostly obtained by precipitation in the presence of organic solvents from culture supernatants and treatment with enzymes such as nucleases and proteases to remove contaminating nucleic acids and proteins [14]. EPS from activated sludge samples can also be extracted by a centrifugation protocol. Then, residual bacteria can be removed from the supernatant by filtration on cellulose acetate membranes (0.2 μm). For removal of low molecular weight material, the supernatants can be dialyzed against deionised water. Depending on the studies, dialysis tubings can have various molecular weight cut-off. Then, dialysate is concentrated by lyophilisation. Other processes of exopolysaccharides extraction from various biofilm species are presented in Table 1.

Biofilm source/ support	Sample preparation	Method of determination	Reference
Pseudomonas fluorescens / polymethyl methacrylate plates	▪ extraction by EDTA 1.5%, 5°C, 3 h ▪ dialyse against deionized water (14 kDa) ▪ precipitation of proteins by pH adjsutement	phenol-sulfuric acid method	Oliveira *et al.,* [17]
Enterobacter cloacae /zinc selenide crystal	▪ extraction by EDTA or NaOH	phenol-sulfuric acid method	Boualam *et al.,* [18]
Leuconostoc mesenteroides strains /stainless steel	▪ scraping and washing biofilm material ▪ extraction by deionized water ▪ centrifugation at 10 000 rpm, 30 min ▪ hydrolysis (endodextranase) ▪ methylation	GC-MS	Leathers and Bischoff [19] Leathers and Cote [20]
Thermus aquaticus YT- /cellophane membrane	▪ extraction by NaCl 0.9% ▪ centrifugation, 8 000g, 15 min ▪ precipitation with alcohol 95 %, 4°C, one night ▪ centrifugation, 2500g, 0°C, 15 min ▪ dissolution in double deionized water and lyophilization	size-exclusion chromatography, GC-MS, HPAEC-PAD*, MS/MS, NMR	Lin *et al.,* [21]
aerobic activated sludge from a wastewater treatment	Comparison of five extraction processes : ▪ extraction by - EDTA 2 %, 4 C, 3 h - cation exchange resin, 4°C, 1 h - formaldehyde, 4°C,1 h - formaldehyde plus NaOH 1 N, 4 C, 3 h - formaldehyde plus ultrasonication (60 W), 2.5 min ▪ high-speed centrifugation 20 000g, 20 min, ▪ filtration (0.2 μm) ▪ dialysis (3,5 kDa), ▪ lyophilization, -50 C, 48 h	anthrone method	Liu and Fang, [16]

*HPAEC-PAD: High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection

Table 1. Extraction condition and determination of exopolysaccharides from various biofilm species

The content of the EPS extracts is done by chemical analyses. The exopolysaccharide content of EPS can be determined by the phenol-sulphuric acid method described by Dubois *et al.* [22] or by using the anthrone method according to Dreywood [23], with glucose as the standard. The protein content of EPS can be determined by the Bradford method [24] or by using the bicinchoninic acid reagent [20] with bovine serum albumin as the standard.

As mentioned by several authors, yields of EPS extracted from biofilms depend on the extraction method used. Pan *et al.* [26] reported that chemical methods significantly increased the extraction yield from natural biofilm compared to physical methods. Nevertheless, the use of chemical methods can modify the composition of the EPS extracted. Indeed, added chemical extractants such as EDTA or formaldehyde can be present in the EPS extracts as contaminants and then can modify the EPS quantification efficiency. Moreover, chemicals can induce the release of intracellular components from treated cells and contaminate extracellular substances by intracellular material. These contaminants may be eliminated.

2.2. Exopolysaccharides of the biofilm matrix

The ability to synthesize exopolysaccharides is widespread among microorganisms, and microbial exopolysaccharides play important roles in biofilm formation, pathogen persistence, and have several applications in the food and medical industries. Exopolysaccharides are considered to be important components of the biofilms matrix [27]. However, some studies suggest that exopolysaccharides may not always be essential for biofilm formation [28]. Most of the matrix exopolysaccharides are very long with a molecular weight of 500-2000 kDa. They can be homo-polymers such as cellulose, curdlan or dextran, or hetero-polymers like alginate, emulsan, gellan or xanthan. Exopolysaccharide chains can be linear or branched. They are generally constituted by monosaccharides and some non-carbohydrate substituents such as acetate, pyruvate, succinate, and phosphate [29]. Various examples of exopolysaccharides encountered in bacterial biofilm are presented in Table 2.

2.3. Carbohydrate content of exopolysaccharides

Composition as well as conformation of sugar monomers may modify the properties of the exopolysaccharides and thus of the biofilm matrix. Mono-carbohydrate constituted exopolysaccharides are often D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, L-arabinose, N-acetyl- D-glucose amine and N-acetyl-D-galactose amine as well as the uronic acids D-glucuronic acid, D-galacturonic acid, D-manuronic acid and L-guluronic acid. Other sugar monomers less frequently occurring are D-ribose, D-xylose, 3-keto-deoxy-D-mannooctulosonic acid and several hexoseamineuronic acids [29]. Some examples of carbohydrate content in biofilm are presented in Table 3.

In conclusion of this section, it is clear that the extraction of exopolysaccharides from biofilms usually require a multi-method protocol. Furthermore, there is no standard extraction procedure established, making difficult the meaning, comparison and interpretation of published results. However, recent studies tend to evaluate whether molecular diversity of EPS are potential markers for biofilm macro-scale characteristics [40].

Exopolysaccharides	Biofilm species	Reference
alginate	*Azotobacter vinelandii* *Pseudomonas aeruginosa* *Pseudomonas fluorescens*	Gorin and Spencer [30] Sabra *et al.,* [31] Donati and Paoletti [32]
curdlan 1,3-β-curdlan-type curdlan-type	*Agrobacterium sp.* ATCC 31749 *Cellulomonas flavigena* *Cellulomonas sp.*	Ruffing and Chen [33] Kenyon *et al.,* [34] Young and Reguera [35]
xanthan	*Xanthomonas citri* subsp. citr	Guo *et al.,* [36]
1,6-α-glucan 1,3-α- glucan glucan	*Streptococcus mutans*	Aires *et al.*, [37]
alternan dextran	*Leuconostoc mesenteroides* strain NRRL B-1355	Cote and Leathers [38]
pyruvated galactan	*Methylbacterium* sp. (isolated from a Finnish paper machine)	Verhoef *et al.,* [39]

Table 2. Examples of exopolysaccharides of bacterial biofilms

Biofilm source/ original source	Carbohydrate nature (concentration)	Method of carbohydrate characterization	Reference
European intertidal mudflat (Marennes-Oléron Bay, France)	galacturonic acid (20%) mannose (19,5%) glucose (19%) arabinose (15%) xylose (8%) galactose (7%)	GC-MS	Pierre *et al.,* [41]
Pseudomonas fluorescence Biovar II	mannose (14%) glucose (<5%) arabinose (28%) xylose (<5%) galactose (45%) fucose (6%) rhamnose (<5%) ribose (<5%)	GC-MS	Hung *et al.,* [42]
Pseudomonas aeruginosa PAO1, PDO300, *alg*D, PA14	mannuronic acid (0-100) % glucose 0-56 % rhamnose (0- 20.7 %) galactose (0-12.4 %) mannose (0-13.9 %) xylose (0-9.7 %) ketodeoxyoctulosonate (0-9.1%) *N*-acetyl galactosamine (0-1.9 %) *N*-acetyl fucosamine (0-7.5 %) *N*-acetyl glucosamine 0-3.8 % *N*-acetyl quinovosamine 0- 18.1% unknown amino sugar 0-5 %	GC-MS	Wozniak *et al.*, [28]
Membrane bioreactor/fouling	uronic acid* glucose* mannose* fructose* xylose* ribose* arabinose* *N*-acetylglucosamine* glucuronic acid* galacturonic acid* maltose* saccharose*	HPLC-SM	Al-Halbouni *et al.*, [43]

(*) not indicated

Table 3. Carbohydrate content of various biofilms

3. Structure and function of different polysaccharides from the biofilm matrix

The most famous exopolysaccharides present inside biofilms are alginate, cellulose and poly-N-acetyl glucosamine. This section focuses on their structures and their function inside biofilms.

3.1. Bacterial alginates

Alginate, a polysaccharide which occurs in brown algae and in different bacteria like *Azotobacter vinelandii* [30] and *P. aeruginosa* [44] has been extensively studied. Alginate is an exopolysaccharide with a relatively high molecular mass (10^4-10^6 g/ml). It consists of the uronic acid residues β-D-mannuronate (M) and its C-5 epimer, α-L-guluronate (G) [45] (Figure 3).

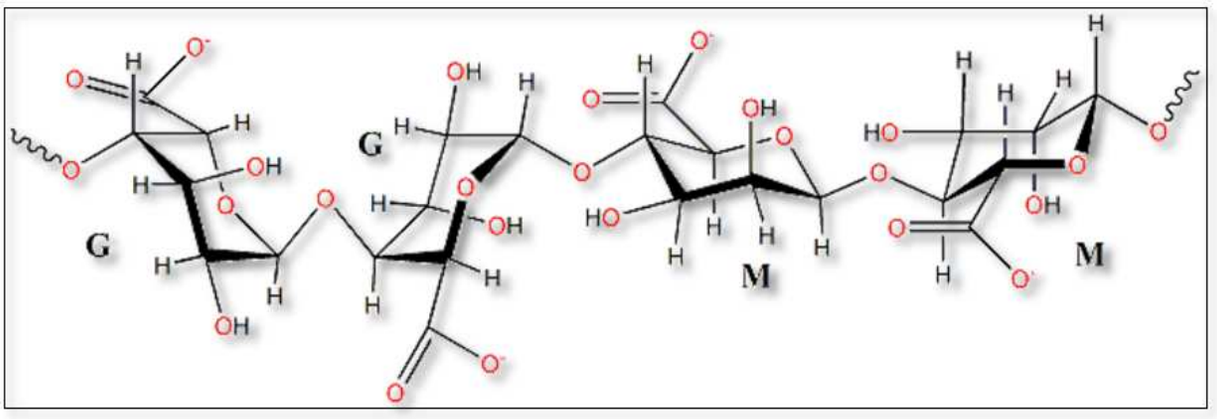

Figure 3. Structure of alginate

Generally, the monomers form a block copolymer with homopolymeric regions of poly-β-D-mannuronate (M-blocks) and poly-α-L-guluronate (G-blocks) as well as heteropolymeric regions (MG-blocks). The absence of G-blocks differentiates alginates produced by *P. aeruginosa* from alginates expressed by algae or by *A. vinelandii* [46]. The functional properties of the alginates strongly correlate with the composition (M/G ratio) and with the uronic acid sequence.

There are 24 genes located on the bacterial chromosome, involved in the production and secretion of alginate in *P. aeruginosa* [45]. Eight genes are implicated in the exportation of alginic acids (Figure 4), and twelve in the biosynthesis of the polysaccharide [47]. The four remaining genes are involved in the regulation of the synthesis.

Alginates can form a gel in the presence of chelating divalent cations. This structure formed is called a Grant "egg-box" [47]. The alginate gel is formed by ionic bonds between the G-rich blocks and divalent cations. The mechanical properties of alginate gels can vary depending on the amounts of guluronic acid present in the polymer. Moreover, alginate gels can be formed in vitro in the presence of proteins such as gelatin [48].

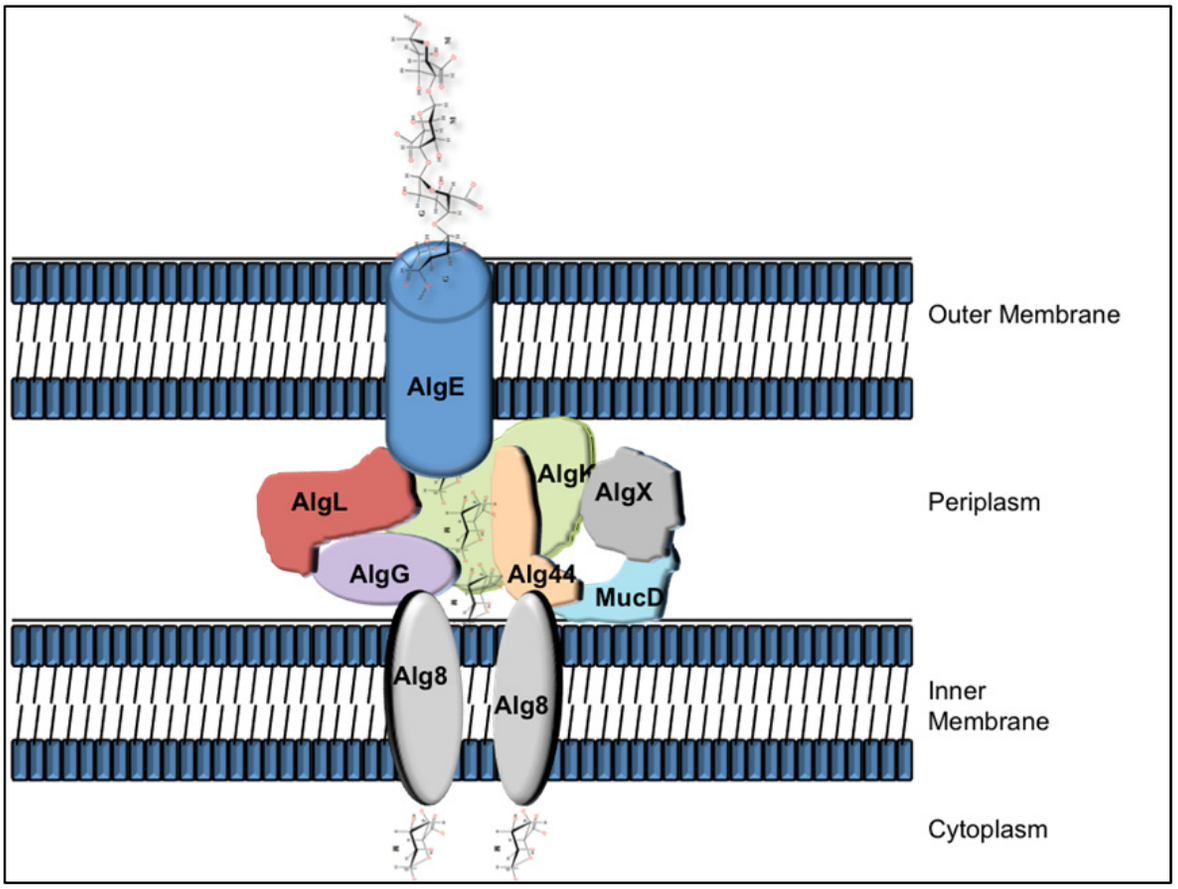

Figure 4. Biosynthesis of bacterial alginate

3.2. Cellulose

Cellulose is the most abundant sugar polymer found on the surface of the planet. It is found throughout the living world: in plants, animals, fungi and in bacteria such as *Salmonella, E. coli, Acetobacter, Agrobacterium* and *Rhizobium* [49].

Salmonella and *E. coli* produce cellulose as a crucial component of the extracellular matrix [50]. Cellulose consists of a β-1-4 linked linear glucose (Figure 5). The formation of cellulose fibers is provided by hydrogen bonds between the chains of glucose. These formed sheets are very stable and their number varies depending on the nature of the environment.

Cellulose has a crystalline structure. Each crystal of cellulose contains numerous glycan chains in parallel orientation. The reducing ends are at one terminus while the non-reducing ends are at the opposite terminus. The structure is not uniform and amorphous regions cohabit with highly crystalline regions.

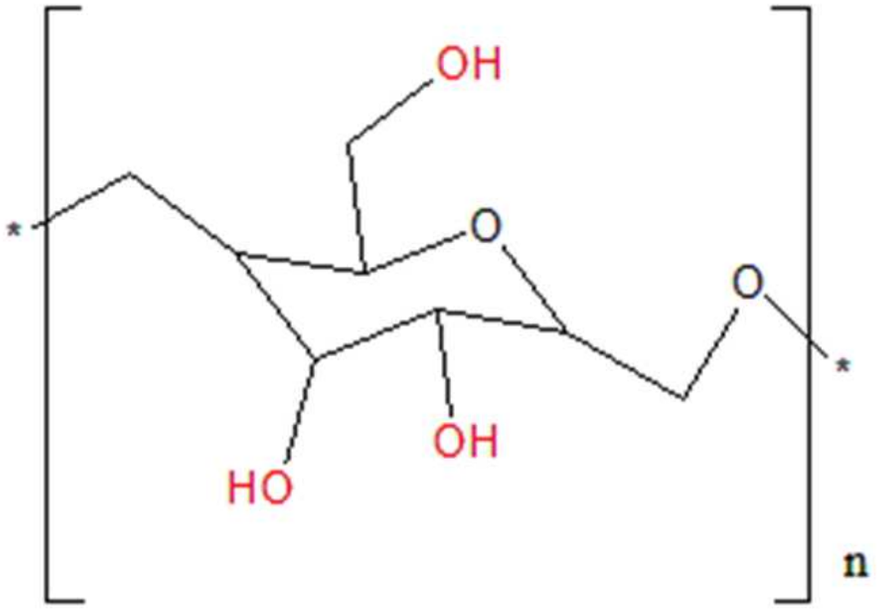

Figure 5. Structure of cellulose

Genes involved in the production of cellulose in *E. coli* and *S. typhimurium* are called *bcs* for bacterial cellulose synthesis (Figure 6). The four *bcs* genes called *bcsA*, *bcsB*, *bcsZ* and *bcsC* are organized as an operon. The *bcs* operon is partially regulated by AgfD, a thin aggregative fimbriae which increases the production of cellulose and curli [49].

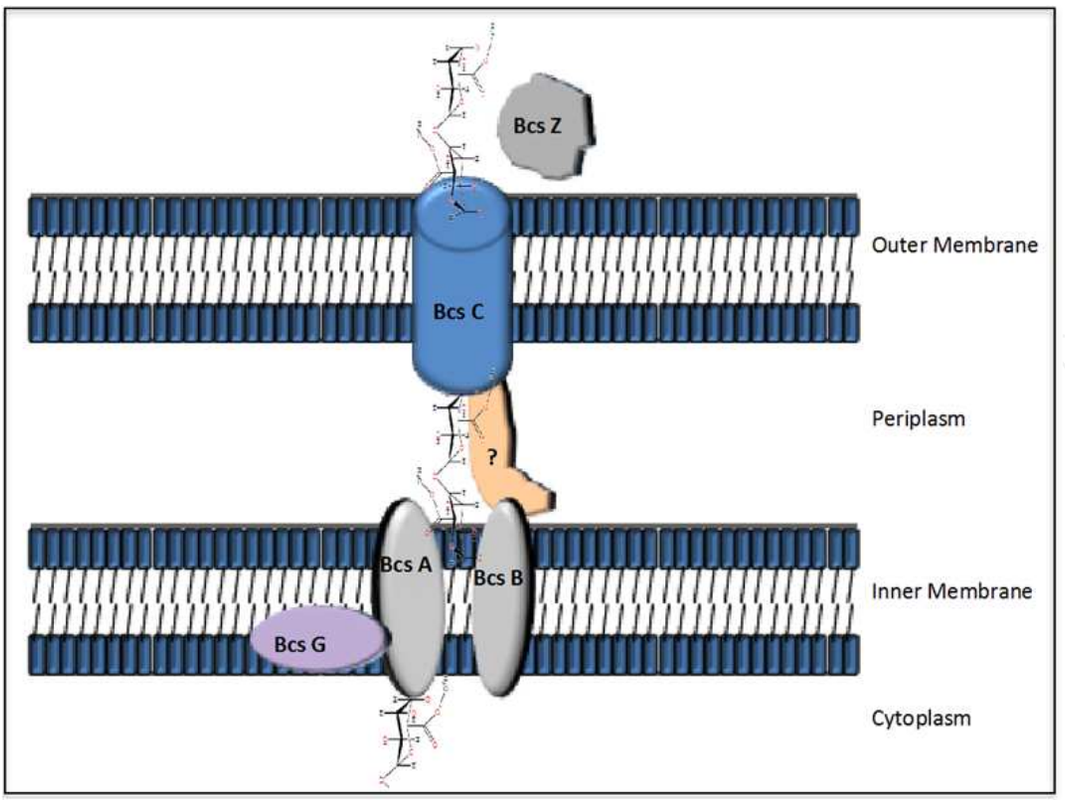

Figure 6. Cellulose biosynthesis

Cellulose can form a gel at adequate temperatures. Cellulose solutions are liquid at room temperature. Gels can form in a cellulose solution at either high temperature (above 50 °C) or low temperature (less than 10 °C). After gelification, cellulose solutions remain more or less stable in the gel state at room temperature [51, 52]. The gel structure of cellulose may explain the mechanical properties of biofilms formed by bacterial species producing this polymer.

3.3. Poly-N-acetyl glucosamine

The polysaccharide intercellular adhesin (PIA) or the related poly-N-acetyl glucosamine (PNAG) polymer is required for bacterial adherence and biofilm formation of some bacterial species. This polysaccharide family was first described in *Saphylococcus* species [53], and further in *E. coli* [54]. PNAG is a positively charged linear homoglycan composed of β-1,6-N-acetylglucosamine residues with approximately 20% deacetylated residues [55] (figure 7).

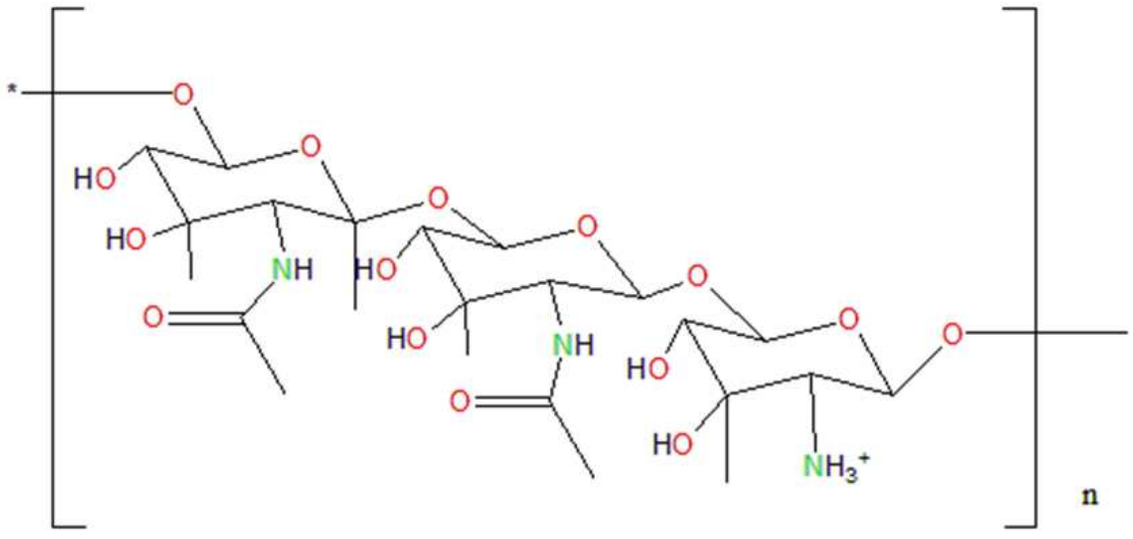

Figure 7. Structure of PNAG

The genes involved in the biosynthesis of PIA are named *ica* for intercellular adhesion. This operon is composed of a regulation gene *icaR* and four biosynthetic genes: *icaADBC* [56, 57] (Figure 8).

PNAG forms a protective matrix around bacterial cells that is also involved in cell-to-cell interactions [53, 54]. PNAG can also interact with eDNA, reinforcing the biofilm matrix structure [58].

3.4. Other polysaccharides involved in biofilm formation

Individual strains or one strain put in different environmental conditions, are able to produce several different extracellular polysaccharides. In mucoid strains of *P. aeruginosa* isolated from patients with cystic fibrosis, mucoidy is due to the overproduction of alginate that is the major constituent of the biofilm matrix. Nevertheless, biofilms formed by non

mucoid strains do not contain alginate. A glucose-rich polymer named PEL (pellicle), and a mannose-rich polymer named PSL (polysaccharide synthesis locus), makes significant contribution to *P. aeruginosa* biofilm structure [59]. The *pel* genes are present in several strains but some commonly used reference strains, such as PAK and PAO1 do not express strongly these genes under common laboratory conditions. The *psl* genes are only present in some strains, but not in the well known PA14 laboratory strain. In some instance, PEL, and PSL can be present together in the biofilm matrix of *P. aeruginosa*.

Other polymers are present in the matrix of the biofilm of *S. epidermidis* for example: teichoic acid [60]. There are two types of teichoic acid in *S. epidermidis*: teichoic acid associated with the bacterial membrane (CW TA) and extracellular teichoic acid (EC TA). The EC TA is responsible for the increased viscosity of the colony. The EC TA is a (1-3)-linked poly(glycerol phosphate), substituted at the 2-position with α-glucose, α-N-acetylglucose, D-alanine and α-glucose-6-alanine (Figure 9).

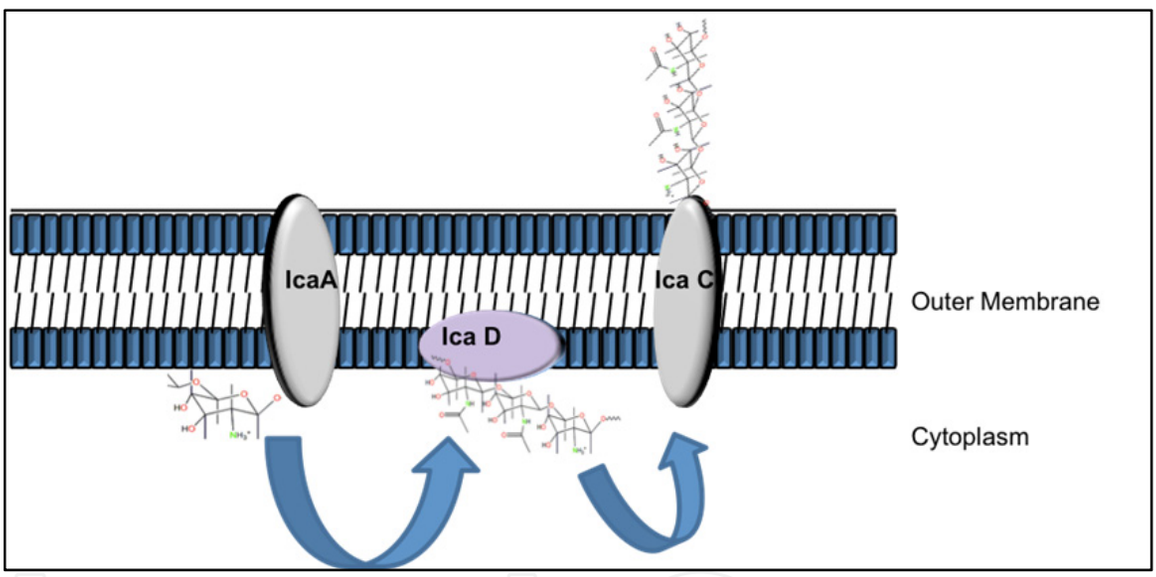

Figure 8. PNAG biosynthesis

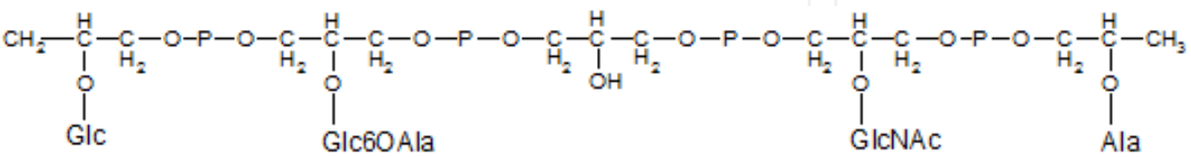

Figure 9. Structure of teichoic acid

In *E. coli*, colanic acid, a sugar polymer composed of galactose, fructose and glucose, is regularly found in the biofilm matrix [61] (Figure 10).

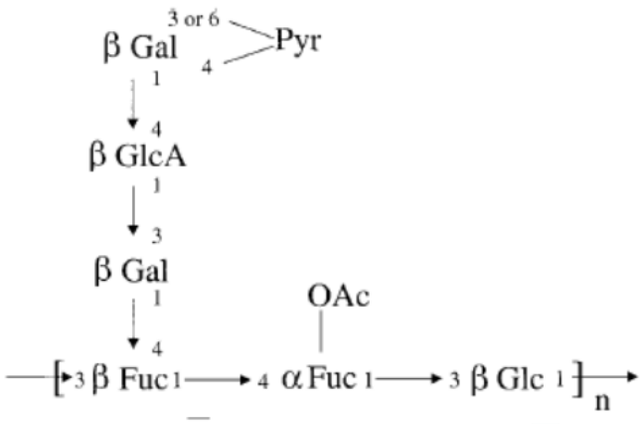

Figure 10. Structure of colanic acid

It must be remembered that although different strains can apparently synthesize the same EPS, there can be differences in physical properties especially with respect to viscosity and gel formation. Several biofilm studies have used colanic acid-producing *E. coli* [61]. Prigent-Combaret, C. et al. [62], yet this polymer can vary greatly in mass and viscosity, as can bacterial alginates.

4. An example of complex biofilm: biofilm formation at the surface of nanofiltration membranes used for drinking water production.

We and others have previously studied very complex biofilms formed on nanofiltration (NF) membranes during surface water filtration in drinking water production processes [63, 64]. After several years of filtration, the foulant consists in a brown viscous layer covering the entire surface of the membrane [65] (Figure 11).

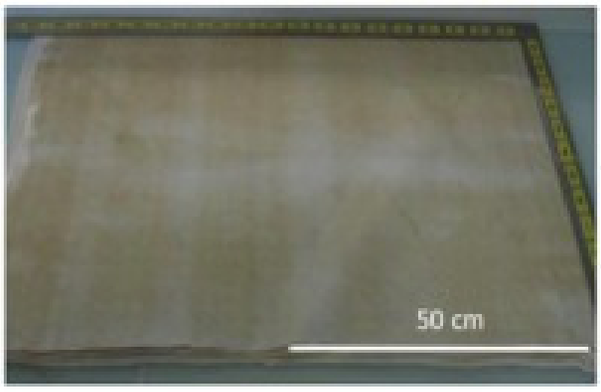

Figure 11. Visual examination of a fouled NF membrane

Dry weight of the foulant is about 2 g/m^2. The NF biofilms harbours mainly exopolysaccharides and proteins, as shown by characteristic ATR-FTIR signals near 1650 cm^{-1} (amide I), 1550 cm^{-1} (amide II), 1450 cm^{-1} (due in part to C-H deformation), 1400 cm^{-1} (due in part to symetric stretch for the carboxylate ion), 1250 cm^{-1} (P=O and C-O-C stretching and/or amide III), and in a broad complex region from 1250 to 900 cm^{-1} (due in part to C-O-

C, C-O, ring-stretching vibrations of polysaccharides and the P=O stretch of phosphodiesters) (Figure 12).

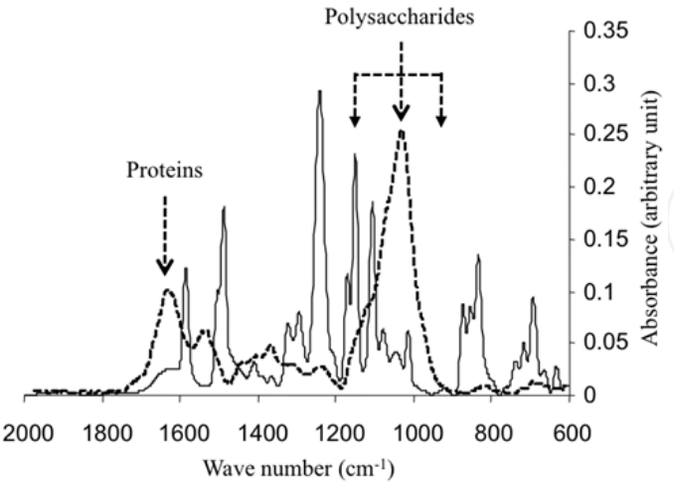

Figure 12. ATR-FTIR spectra of a virgin membrane (plain line) and of a fouled membrane (dotted line)

Fluorescence microscopy observations after nucleic acid staining with DAPI and polysaccharides staining with lectins labelled with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate indicate a high spatial heterogeneity inside the foulant matter with a mean thickness of 32.5 ± 17.7 μm [66] (Figure 13). Examples of lectins that can be used for such polysaccharides staining experiments are peanut agglutinin (PNA) targeting β-gal(1->3)galNAc residues, wheat germ agglutinin (WGA) targeting $(glcNAc)_2$ and NeuNAc residues, *Bandeiraea simplicifolia* (BS-1) agglutinin targeting α-gal and α -galNAc residues and Concanavalin A (ConA) targeting α-man and α-glc residues.

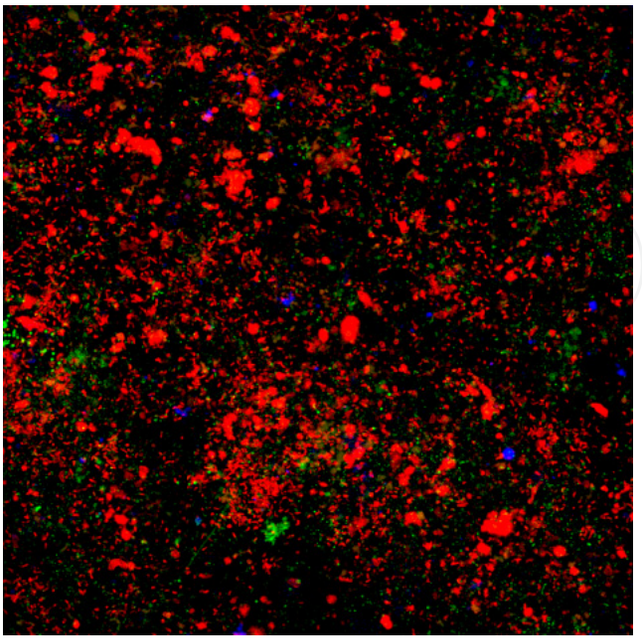

Figure 13. CLSM visualization of the heterogeneity of a NF biofilm after staining with DAPI, TRITC and TITC-labelled lectins. Magnification x630

The microbial cells, mainly composed of bacteria, are localized in the superficial layer of the fouling material and are organized as microcolonies interspersed at the membrane surface. Some algae are also present, as shown by autofluorescence properties. The presence of a dense and wide polysaccharide matrix harbouring few microbial cells at the NF membrane surface has been associated with differences in the efficiency of cleaning procedures against different foulants categories [65, 67]. Polysaccharide residues are found in areas where microcolonies are present and in areas devoid of microbial cells. This polysaccharide organization has been previously observed with environmental biofilms grown in vitro with river water as the sole source of carbon and nutrients [68]. High staining with PNA and BS-1, respectively reveals high occurrence of galactosides residues in the polysaccharide components of the foulants. The BS-1 lectin staining pattern indicates a high degree of spatial organisation with the observation of long and entangled fibers. WGA staining shows short fibers and cloud stained areas. PNA and ConA lectin staining are more interspersed. The polysaccharide composition of the fouling layer changes quantitatively and qualitatively during spring and summer [64]. Lectin staining increases from March to September for all the lectins used. Staining with BS-1 increases constantly in March, June and September. A high increase of binding with PNA, and ConA is observed between March and June, but the binding of these two lectins does not change between June and September. Staining with the WGA is weak in March and June and is higher in September. The lectin-binding changes with time may be linked to an increase of the biomass attached at the membrane surface and to changes among the populations of attached cells. Nutrients, oxygen level and the concentration of metals can influence the exopolymer abundance of environmental model biofilms grown in vitro with river water as the sole source of carbon and nutrients [69]. The modification of these parameters leads to a shift in the glycoconjugate makeup of the biofilms.

Biofilms may be considered to be highly porous polymer gels [70] and diffusion studies demonstrate gel-like characteristics [71]. Previous work has suggested that laboratory-grown and some natural biofilms are viscoelastic in nature [3, 8, 72]. During rotation analysis, a rheofluidification behaviour is observed for NF biofilms [66]. Different mechanisms can explain shear thinning of a biofilm. Break down of links between polymers in the biofilm matrix or deflocculation of particles corresponding to an irreversible modification of the biofilm structure can occur. Such irreversible modifications are unlikely in the experimental conditions published because of the reversibility of viscosity changes with shear rate [66]. Shear thinning of NF biofilms may be related to the polymeric composition of the biofilm matrix. With shear acceleration, polymers may follow the direction of the flow leading to viscosity decrease. This has been previously observed with purified polysaccharides like cellulose [73]. Moreover, bending of biofilm structures in the shear direction during the application of shear stress has been mentioned to explain the viscoelastic response of a mixed culture biofilm [72]. NF biofilms have been submitted to oscillation analysis with a cone-plate rheometer [66]. In such experiments, a sinusoidal oscillation of defined maximum strain and oscillatory frequency is applied to a sample and the storage (G') and lost (G'') modulus are measured. The storage modulus characterizes the

ability of the material to store energy, whereas the loss modulus characterizes energy dissipation in the material under dynamic excitations. If the material is perfectly elastic then the resultant stress wave is exactly in phase with the strain wave. By contrast, when the rate of change of the sinusoidal oscillation is a maximum and the strain is zero, for purely viscous systems, the resultant stress wave will be exactly 90° out-of phase with the imposed deformation. For NF biofilms, during oscillation analysis, values of storage modulus (G') stay higher than values of loss modulus (G'') over the entire range of frequencies covered, indicating that the NF biofilm behave like a highly elastic physical gel [74]. Polysaccharides alone, like alginates, are known to realize a sol-gel transition under adequate physicochemical conditions [75]. The physicochemical microenvironment inside NF biofilms may be permissive to exopolysaccharides sol-gel transition. The gel state is resistant enough and presents a micro porosity favourable for resistance to flow forces, microcolonies development and cell nutrition inside the biofilm structure. This model of sol-gel transition of polysaccharides inside biofilms is consistent with rheological properties previously demonstrated for other biofilms: *Streptococcus mutans* biofilms have elasticity and viscous behaviour analogous to NF biofilms for a range of frequencies between 0.1 to 20 Hz [76]. The rheofluidification behaviour and gel-type rheological properties shared by different type of biofilms and purified polysaccharides suggest that the critical components of the biofilm matrix determining the biofilm texture are polysaccharides.

The time-dependent strain response observed in the creep curves clearly indicated that NF biofilms exhibited viscoelastic behaviour. Viscoelasticity is thought to be a general mechanical property of biofilms. A very wide range of elasticity and viscosity values has been previously observed for a wide sample of biofilms formed artificially in laboratory experiments or coming from natural aquatic environments [4, 72, 76]. Thus, it wasn't surprising to observe that the rheological properties of NF biofilms are different from the ones of natural biofilms from different aquatic environments like Nymph Creek (Yellowstone National Park) and Chico Hot springs (Montana) algal biofilms [4]. These differences in viscosity and elasticity between biofilms can be related to different exopolysaccharide contents and to different shearing strains. Bacterial and algal alginates are known to have different monomeric composition leading to a stronger binding of cations for bacteria, a property involved in the formation of a stable gel in the presence of ambient Ca^{2+} cations [77].

The specificity of NF biofilms may be the necessity to resist shear forces applied to the membrane during the filtration process. In the Méry-sur-Oise plant, NF membranes are operated at feed pressure of approximately 10 bars [78]. The high membrane feed pressures may influence the rheological properties of NF biofilms by increasing cohesive forces in the biofilm bulk, increasing forces, which keep the exopolymers to the membrane surface, and thus strengthening the mechanical stability of the biofilm. This may explain at least in part the NF biofilms resistance to industrial cleaning [65].

Shaw et al. have previously shown that the elastic relaxation time varied much less between biofilms of different origins. λ was estimated to be the time required for viscous creep length to equal elastic deformation length (so that memory of initial conditions is lost), i.e., $\lambda \approx \eta / G$.

The elastic relaxation time of about 30 minutes lies within the range previously determined for various biofilms [4]. The universality of the viscoelastic transition of biofilms has been suspected to have critical survival impact [4]. The ability of biofilms to deform in response to mechanical stress may be a conserved strategy of defence to enable persistence on surfaces in different flow conditions.

5. Conclusion

Extracellular polysaccharides are considered as the major structural components of the biofilm matrix. A large variety of polysaccharides required for bacterial adherence and biofilm formation have been described. Polysaccharide molecules can interact with themselves or with ions and proteins. These interactions result from electrostatic attractive forces, repulsive forces, hydrogen bonds, van der Waals interactions and ionic attractive forces. All these forces influence the structure and the stability of the biofilm matrix and the way EPS and polysaccharides can be extracted from the biofilm bulk. A universal protocol for extracellular polysaccharide extraction from the biofilm matrix does not exist. Each study may adapt usual extraction procedures to biofilm specificities and to the nature of the polysaccharide studied. The viscoelasticity nature of biofilms is universal but biofilms in differing environments exposed to different hydrodynamic conditions will encounter changes in the structure, composition and then physical properties of their matrix. Biofilm science is highly exciting since it is a mixture of biology, biophysic, chemistry and much more.

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