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Review

Biosynthesis of Extracellular Polymeric Substances (EPS) and Its Role in Microbial Biofilm Formation

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Abstract

Microbial biofilm formed on abiotic surfaces is an important area of research because of the wide range of possible affects and the disinfectant resistance of the cells. The colonization of solid surfaces by microorganisms is a very complicated process that depends mostly on extracellular molecule production. The biosynthesis of EPS reflected not only the attachment and aggregation process but also provided an optimal environment for the exchange of genetic material between the cells. The comparative and comprehensive analysis of all documented data concerning EPS production can enable the development and effective control strategies for biofilms. In this review some of the basic concepts concerning the biosynthesis of EPS and potential function of these compounds in biofilm development were discussed. In the paper the positive and negative aspects of EPS production in the environment also were described.

Keywords: extracellular polymeric substances, EPS, biofilm, adhesion

Introduction

Microbial biofilms play a crucial role in a variety of disciplines, including biotechnology, immunology, biofouling and biodeterioration [1, 2]. Literature includes some evidence that cell contact with surfaces stimulates transcription of the EPS genes [2]. Monitoring the EPS gene expression in adherent populations enables a better understanding on the basis of biofilm phenotype. [3-5].

The biosynthesis of EPS is believed to serve many functions concerning: promotion of the initial attachment of cells to solid surfaces; formation and maintenance of microcolony and mature biofilm structure; and enhanced biofilm resistance to environmental stress and disinfectants. In some cases, EPS matrix also enables the bacteria to capture nutrients [2, 6].

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The production of EPS by attached microorganisms is a very complicated process, which is affected by many unique parameters. It is also considered that the mechanisms of biofilm development process are vastly different from species to species [2]. Despite the difficulties associated with the study of the production of EPS by anchored cells, analysis of all described data can enable control of the microbial adhesion process in different environments.

Composition of EPS

The EPS matrix is generally from 0.2 to 1.0μm thick. In some bacteria species the thickness of the EPS layer does not exceed values from 10 to 30nm [7]. The chemical structure of polymeric substances secreted by the cells into the environment is diversified. EPS compounds belong to such different classes of macromolecules as polysaccharides, proteins, nucleic acids, glycoproteins

and phospholipids [8, 9]. Among one bacteria species, EPS compounds may also belong to different categories. These microorganism features are often used during cell identification and classification procedures. In addition the usage of the antigenic properties of the extracellular molecules enables the serological characterization of the cells.

Most microbial exogenous layers contain neutral carbohydrates (mainly-hexose, seldom-pentose) and uronic acids. The commonest extracellular carbohydrates substituents are acetate esters, pyruvtes, formates and succinates. The presence of polypeptides in the EPS matrix is the feature of a very few Gram-positive bacteria cells. The best-investigated components of the EPS layer are polysaccharides and proteins [7, 8].

The structures of polysaccharides synthesized by microbial cells vary greatly in their kinds of linkages and non-sugar substituents [10]. It was observed in the cells of *Sinorhizobium* spp., *Leuconostoc* spp. and *Streptococcus* spp. [11]. Microbial exopolysaccharides are comprised of either homopolysaccharides or heteropolysaccharides [12, 13]. Homopolysaccharides are composed of only one monosaccharide type: D-glucose or L-fructose. Homopolysaccharides belong to three distinct groups:

– α-D-glucans produced by *Leuconostoc mesenteroides*. These compounds contain mostly α(1→6) linked D-glucosyl units. The degree of branching involves α(1→3) linkages, seldom α(1→2) and α(1→4) linkages;
– β-D-glucans, synthesized by *Pediococcus* spp. and *Streptococcus* spp. The molecules are composed of β(1→3) linked D-glucosyl units with branching involving β(1→2) linkages;
– fructans produced by *Streptococcus salivarius* containing β(2→6) linked fructosyl units [8].

A number of lactic acid bacteria produce heteropolysaccharides. These molecules formed from repeating unites of monosaccharides such as: D-glucose, D-galactose, L-fructose, L-rhamnose, D-glucuronic acid, L-guluronic acid and D-mannuronic acid. The type of both the linkages between monosaccharide units and the branching of the chain determines physical properties of microbial heteropolysaccharides. Most heteropolysaccharides also possess substituents of pyruvtes, succinates and formates [8, 12]. Bacterial alginate is a heteropolysaccharide with irregular structure. In this polymer, D-mannurosyl and L-guluronosyl residues are found. Alginate is produced mostly by the cells of *Pseudomonas aeruginosa* and *Azotobacter vinelandii* [8].

Extracellularly secreted proteins are substances with molecular masses between 10kDa and 200kDa. These compounds contain from 40% to 60% of hydrophobic amino acids. Generally in the structure of exogenous proteins the lack of sulfuric amino acids was noticed. It was especially observed in the cells of *Geobacillus stearothermophilus*. Extracellular proteins synthesized by *Sulfolobus acidocalcidarius* are composed mostly of amino acids with hydroxyl groups [14]. However, the *Bacillus subtilis* extracellular protein layer is a composition of L- and D-glutaminosyl residues. Under oxygenate conditions in the structure of these extracellular compounds the ratio of L- to D-glutaminosyl residues equals 1. According to Ton-That et al. [15] the ratio of glutaminosyl isomers in the *Bacillus subtilis* extracellular protein layer changed significantly in oxygen-limited conditions.

Physiological Determinants of EPS Biosynthesis

Effect of Carbon/Nitrogen Availability

The extracellular biopolymers' synthesis by microbial cells depends on the carbon and nitrogen availability in the culture medium. Most exopolymer-producing microorganisms utilize carbohydrates as their carbon and energy source and either ammonium salts and amino acids as their source of nitrogen [16-18].

In general EPS production increased under conditions where growth was extended by the high glucose content in the medium [1]. The biosynthesis of extracellular compounds in *Acetobacter xylinum* cells might be determined by the availability of fructose, sucrose and starch in the medium at the level between 25 and 100g/l. The lowest efficiency of EPS molecule production occurred under galactose and xylose availability in the growth environment [8]. Carbohydrates, such as: glucose, fructose, mannose, maltose, xylose, ribose, arabinose, sucrose and lactose, also determine the extracellular polymeric substances production in *Aureobasidium pullulans* cells. The highest efficiency of this process was noticed upon 70% of carbohydrate content in the culture medium [19].

Low nitrogen content in the growth environment also influences the extensive microbial synthesis of extracellular biopolymers [7]. Under limited ammonium salts availability in the medium, 60% of the glucose was converted into exopolysaccharides in the strains of *Aureobasidium* spp., *Sinorhizobium* spp., *Escherichia* spp. and *Pseudomonas* spp. [8, 19]. The high content of nitrogen sources in the medium induces extracellular protein production by microbial cells. Sanin et al. [20] observed the increasing biosynthesis of exogenous proteins in *Pseudomonas* spp. and *Rhodococcus* spp. cells incubated upon the high ammonium salts available in the medium.

pH Value of Culture Medium

The pH value of the culture medium significantly influences EPS molecule production. This parameter determined the morphological changes of the cells. The extreme pH profiles of the medium (pH 2.0-3.0 or pH ≥ 10) inhibited not only the process of microbial growth but also the biosynthesis of extracellular polymers [21, 22]. It was particularly observed in the cells of *Aureobasidium pullulans*. Lee et al. [19] performed the minimum productivity of the EPS compounds related with morphological

changes of *Aureobasidium* cells grown at a low pH value of 2.0 of the medium. Generally the optimal pH profile of the medium for the EPS production oscillated between 5.5 and 6.5 [19].

The pH value of the growth environment might also be a stimulated factor of the EPS molecules production by *Antrodia camphorate* cells. Shu and Lung [23] showed the highest productivity of these extracellular compounds, using the medium with the pH value of 5.0. Both increasing and decreasing pH value of the culture medium significantly inhibits extracellular polymer biosynthesis by *Antrodia* spp. cells. According to Shu and Lung [23] the pH profiles manipulation of the culture medium also influences molecular mass of the EPS compounds.

Cultivation Temperature

The effect of the cultivation temperature on the exogenous proteins and exopolysaccharides biosynthesis by microbial cells also was investigated. Generally, the optimal cultivation temperature for the production of most EPS molecules was estimated between 26 and 31°C [14, 16]. This dependence also was confirmed by the results of Gancel and Novel [24] concerning the optimization of EPS compound production by *Streptococcus salivarius* cells.

According to Sutherland [8] reduction of the cultivation temperature by 10°C below optimal level inhibits the exopolysaccharides biosynthesis by microbial cells. However, under low temperature of the growth environment profiles of the high productivity of extracellular proteins by bacteria cells might be observed. It was noticed in *Listeria* spp. cells. Briandet et al. [25] showed that a cultivation temperature of 10°C induced in extracellular cold shock protein production *Listeria monocytogenes* cells.

Table 1 lists the major physiological determinants required for the highest productivity of EPS compounds by particular microorganisms.

Growth Phase

Dependence between EPS production and the stage of the microbial growth cycle is a feature of particular genera. In strains of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, high productivity of the EPS molecules was observed during the late logarithmic and early stationary phases of microbial growth [8, 26].

Literature shows clear evidence that the rate of extracellular polymers synthesis by *Pseudomonas* spp. and *Escherichia* spp. is determined by the proliferating process of cells. The ceasing of the exponential growth phase induces losing the integrity of the microbial cell surface. This process is caused by the reduction or even lack of extracellular molecules production by microorganisms [14, 27]. Similar dependences were observed during the investigation of the relationships between the stages of *Enterobacter aerogenes* growth cycle and the EPS molecules biosynthesis [2]. At the end of logarithmic growth phase there was no production process noticed of extracellular compounds by *Enterobacter aerogenes* cells.

Molecular Aspects of EPS Biosynthesis

The regulations mechanisms of the EPS production has not yet been well defined. Enzymes needed for the formation of EPS precursors, appeared to be under separate control from mechanisms of gene expression associated with the EPS molecules biosynthesis [8]. The exceptions are the xanthan and extracellular protein production by

Table 1. Major environmental conditions required for the highest productivity of EPS components by microorganisms.

Physiological determinant	Species	References
Glucose (2%) Fructose (2%) Mannose (2%) Maltose (2%) Xylose (2%)	*Aureobasidium* spp. *Bacillus* spp.	[16, 19]
Sucrose (2.5-10%)	*Acetobacter* spp.	[8]
$(NH_4)_2SO_4$ (0.04-0.06%)	*Aureobasidium* spp. *Sinorhizobium* spp. *Escherichia* spp. *Bacillus* spp.	[8, 16, 19]
pH 5.0-6.5	*Aureobasidium* spp. *Antrodia* spp.	[19, 23]
Temperature 26-31°C	*Bacillus* spp. *Streptococcus* spp. *Lactobacillus* spp. *Pseudomonas* spp. *Acetobacter* spp.	[14, 16, 24]

Xanthomonas campestris cells. Tang et al. [28] performed that only one region of the *Xanthomonas* spp. genome (*rpf*) leaded to both the EPS molecules production and the control of enzyme biosynthesis needed for the EPS precursor transformations. The *rpf* genes controlled extracellular enzymes, including cellulase, polygalacturonate lyase, amylase and protease.

Under extreme environmental conditions, microorganisms differing taxonomically may produce the same or almost identical types of extracellular compounds. It was observed in the strains of *Pseudomonas* spp. and *Azotobacter* spp. [8]. The studies of Boucher et al. [29] and Mejiaruiz et al. [30] concluded that in *Pseudomonas aeruginosa* and *Azotobacter vinelandii* cells, the processes of biosynthesis and secretion of extracellular molecules were controlled by a similar gene cluster. This gene cluster includes: *algA* gene encoding GDP-mannose pyrophosphorylase, *algD* coding GDP-mannose dehydrogenase and *algE* coding a membrane protein probably involved in alginate export. A separate location in the bacterial chromosome of additional genes involving in the alginate expression process also was found. This feature was detected only in *Pseudomonas* spp cells. It is considered that these genes enable the differentiation between *Pseudomonas aeruginosa* and *Azotoacter vinelandii* cells. To these genes belong *algC* gene coding phosphomannomutase and *algK* gene with unknown function. The studies of Aarons et al. [31] and Jain and Ohman [32] performed the defect of the *algK* gene blocked the alginate biosynthesis reactions in *Pseudomonas aeruginosa* cells.

In a number of bacteria species, the EPS synthesis is controlled through megaplasmids rather than chromosomally. It was particularly observed in the strains of: *Escherichia* spp., *Streptococcus* spp. and *Lactobacillus* spp. [8]. The regions contained a cluster of genes involved in EPS molecule production could be replaced by equivalent segments from other bacteria strains. According to Gacesa [33] this leads to the appearance of a similar cluster of genes and their products in *Pseudomonas aeruginosa* and *Azotobacter vinelandii* cells.

Role of EPS in Biofilm Formation

Importance of Microbial Adhesion Process

The EPS molecules are regarded as the major factor influencing the microbial biofilm formation process. The extracellular compounds promote more developed stage of cell attachment processes, the so-called specific adhesion phase or irreversible adhesion phase. The production of the extracellular polymeric substances occurs more extensively during the specific adhesion stage. EPS molecules strengthen the interactions between the microorganisms and as a result they determine the cell aggregates formation process on the solid surface [9, 34, 35]. Recent reports suggest that mostly extracellular proteins, exopolysaccharides and extracellular DNA are responsible for the architecture and morphology of the biofilm matrix [36, 37].

The extracellularly secreted proteins determine the microbial attachment process to different solid surfaces [34, 38]. Firstly these macromolecules are accumulated on the cell surface. After the secretion process to the external environment, the proteins may be adsorbed to contact surfaces [39, 40]. A protein layer formed on solid surface is conducive to bacterial adhesion process. The layer of adsorbed proteins might convert the solid/medium interface into a region of gel-like nature which other specific polymers of the bacteria surfaces can interact with [1]. During more advanced phases of the microbial adhesion process, the *in situ* secretion of extracellular proteins might also be observed. This leads to the intensification of the microbial attachment process by anchoring the single cells on the contact surface [41, 42]. The relationship between the protein layer and the microbial adhesion process refers to van der Waals interactions, electrostatic forces and hydrophobicity of surfaces [2, 43]. The adsorption properties of extracellular proteins and contact surfaces base firstly on the interfacial redistribution of charged groups and, secondly, on the hydration changes of proteins, cell surfaces, and contact surfaces. The microbial attachment process on the solid surfaces is also affected by the tertiary protein structure and the molecular interactions between conjugative pili and contact surfaces [41, 44].

Literature shows clear evidence that the biosynthesis of extracellular proteins plays a significant role in the microbial colonization process. Jenkinson [45] performed the *Streptococcus oralis* adhesion to the teeth surfaces determined by adhesive protein expression. Similar dependences were noticed during the investigation of the attachment process of *Azospirillum brasiliense* to the surface of glass and polystyrene [46]. Elimination of the outer protein layer from the cells by trypsin or SDS solutions caused significant (even 100 times) reduction of *Streptococcus* spp. and *Bacillus* spp. adhesion to the stainless steel surface [47, 48]. The studies of Neu [38] and Ahimou et al. [49] concluded that decreasing production of extracellular proteins by microorganisms induced the changes of cell surface properties (e.g. hydrophobicity).

Exopolysaccharides have been termed "adhesive polymers." These macromolecules are believed to be important factors determining the microbial biofilm formation process on the solid surfaces [36, 38, 50]. Chen and Stewart [51] suggested that extracellularly secreted polysaccharides are responsible for both adhesion and cohesion interactions and play a crucial role in maintaining structural integrity of biofilms. It is worth pointing out that the content of polysaccharides is at least fivefold higher than extracellular proteins in biofilm [52]. Some authors also considered that exopolysaccharides can promote a preconditioning of the surface, making the adhesion process more favorable [53]. The studies of Parkar et al. [48] concluded that elimination of the exopolysaccharides layer from the cells by trichloroacetic acid or lysozyme solu-

tions decreased *Bacillus* spp. attachment to stainless steel surfaces by approximately $\log_{10}$ counts cm^{-2} of 0.5 – 1.5. However, a lack of correlation between exopolysaccharide productivity and microbial adhesion process to the solid surfaces was noticed during the investigations. Similar dependence of the attachment process to abiotic surfaces of non-polysaccharide-producing mutants as well as polysaccharide-producing cells were observed by Allison and Sutherland [3].

The biosynthesis of extracellular polysaccharides can be stimulated by organic acid availability in the culture medium. However, these carbon sources decrease flocculation mechanisms of bacteria cells. The effect of the competition mechanisms between carboxyl groups of polysaccharides and aliphatic acids can be balanced by adding the calcium ions to the medium. The promotion of exopolysaccharides biosynthesis by organic acidrich mediums is considered to be the feature of the catabolic repressions mechanisms (glucose also evidently represses that biosynthesis) [54]. Also, limited nutrient availability in the medium influences the extensive microbial synthesis of exopolysaccharides. However, control mechanisms of that process have not yet been described [1].

The aeration, flow rate and detachment force stimulate extracellular polysaccharide production. These macromolecules determine the biofilm matrix stability [52]. The highest productivity of these compounds is observed during the early stages of the biofilm formation process [2]. This feature promotes the initial cell adhesion to the solid surfaces [18, 52]. In literature there is some evidence that exopolysaccharides can play a crucial role in building up three-dimensional biofilm structure. The correlation between production of these exopolysaccharides and biofilm density was noticed by Tsuneda et al. [55]. It was also performed that the detachment forces may lead to a thinner and denser biofilm. These interactions are physical rather than biological [56].

Extracellular DNA has recently been described as one of a major structural components of the *Pseudomonas aeruginosa* and *Streptococcus mutans* biofilm matrix [5, 57]. However, the role of this extracellular molecules in the Gram-positive and Gram-negative biofilm development process is still unclear. Whitchurch et al. [57] reported that attached *Pseudomonas aeruginosa* cells produce substantial quantities of extracellular DNA through a mechanism that is independent of cellular lysis. Releasing these macromolecules by the sessile bacteria induce the formation of strengthening linkages between the cells in biofilm structure. According to Petersen et al. [5] the presence of extracellular DNA in the *Streptococcus mutans* biofilm matrix is associated with the export of a large quantity of competence-signaling peptides (CSP) to the medium. Authors have shown that exogenous DNA support the horizontal gene transfer of naturally competent bacteria in the mature biofilm structure. The horizontal gene transfer within biofilms directly determined the antibiotic resistance of the attached cells [58].

Importance of Biofilm Resistance to Antimicrobial Agents

Bacterial cells undergo a number of physiological and phenotypic changes following attachment to a solid surface. These lead to higher cell resistance to antimicrobial agents. Bacteria living in biofilms can be up to 1000 times more resistant to antibacterial compounds (such as disinfectants, antibiotics, surfactants) than planktonic cells [59, 60]. Recent evidence suggests that the EPS matrix surrounding the attached cells provides an effective barrier that restricts penetration of chemically reactive biocides inside the biofilm [61]. Both structure and properties of extracellular compounds associated with solid surface cells differ from those synthesized by planktonic bacteria [62]. These differences refer mostly to polysaccharide components of the EPS layer. The expression of *algC* gene coding extracellular alginate is activated only during the biofilm formation process by *Pseudomonas aeruginosa* cells. It occurs after 15 minutes following the attachment of cells to solid surfaces [63]. The alginate synthesis induces the hydrophilic properties of *Pseudomonas aeruginosa* cell surfaces. The studies of Nichols et al. [64] and Sauer and Camper [65] performed that increasing hydrophilic value of bacteria cell surface restricted penetration of antimicrobial agents with hydrophobic character. In addition, Gordon et al. [66] noticed that high productivity of negatively charged extracellular alginate significantly delayed the transport rate of aminoglycosides through biofilm structure. According to Hentzer et al. [67] the extracellular alginate overproduction also increased the tobramycin resistance of *Pseudomonas* spp. biofilm compared to planktonic bacteria.

In the literature there are clear evidences that the high range of polysaccharide components increased the amount of functional groups in the EPS matrix. It determines a lower susceptibility of biofilm populations to biocydes, cationic antibiotics and antimicrobial peptides [62]. The functional groups of exopolysaccharides react with antimicrobial agents. It prevents the diffusion process of toxic compounds to cytoplasm. However, it is recognized that killing properties of antibiotics are increasing while all possible binding sites in the EPS matrix are becoming saturated. Therefore, the microbial biofilm resistance to toxic compounds significantly decreases after long-term exposure [61].

Relevance of EPS in the Environment

The influence of EPS compounds on microbial biofilm formation process is an important area of research because of the wide range of possible affects and the disinfectant resistance of the cells. From a medical perspective, the attached bacteria on catheters, drains, implants or lenses are of the greatest concern because they can cause serious infections [68]. The extracellular polymeric substances surrounding the attached cells restrict antibiotic penetration.

This feature causes difficulties in treatments. Microbial biofilm formation on teeth surfaces leads to tooth decay and paradentosis [69].

For the food industry in particular, the formation of biofilms on food contact surfaces constitutes an increased risk of product contamination with spoilage or pathogenic microflora. The microbial cells anchored on solid surfaces by EPS matrix are difficult to overcome by disinfectants. From a hygienic perspective the effect of tearing the cell aggregates away from the mature biofilm structure is also a problematic issue. In general there is no surface material which cannot be colonized by microorganisms. The EPS compounds promote cell attachment to the surface of heat exchangers or a surface of piping systems transferring the media [56]. In some cases, biofilm leads to significant losses in performance and increasing the energy demand in heat exchanger technology [70, 71].

The EPS molecules are also involved in microbially corrosion of metal surfaces. It was particularly observed on ship hulls and in piping systems [72]. The corrosion reactions caused by EPS matrix produced by attached cells refer also to wood, concrete and plastic materials [73].

The microbial adhesion process to solid surfaces also has beneficial consequences. The species of *Pseudomonas putida* prevents stainless steel materials from corrosion [56]. The cells of *Pseudomonas* spp. are capable of binding the phosphate ions on stainless steel surfaces. Recent evidence suggests that this process remains stable even after elimination of the biofilm matrix [74].

Environmental applications of the EPS compounds have been focused so far on the degradation process of organic substances, the denitrification of wastes, phosphate ion elimination from manufacturing and municipal wastes. The studies of Skłodkowska and Matlakowska [75] showed that the extracellular substances also bind heavy metals from different environments.

The usage of EPS compounds in the food industry have been intensively investigated. The novel properties of microbial exopolysaccharides such as xanthan, curdlan, pulluan and alginate, may improve food viscosity, hydration of products and low calories food production. It is also considered to apply the microbial extracellular polysaccharides for food edible coating production that effectively would protect products from spoilage [76, 77].

The study of EPS excretion by attached microorganisms and its role in the development of biofilm is very difficult to conduct. In fact the true structure of adhesive polymers and which of the EPS molecules are particularly involved in maintaining biofilm architecture are still uncertain. It is also unclear how long the cells within the biofilm matrix excretes EPS molecules [8]. A better understanding of the factors affecting the biosynthesis of extracellular polymers and its role in the biofilm development process will help in the eradication of attached bacteria from surfaces. It also will create capabilities in the environmental use of the attached microorganisms [38].

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