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Review article

# Biofilms – a danger for food industry

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***Abstract:*** *Bacterial biofilm is a complex structure of microorganisms with variable qualitative composition depending on the raw materials used, parameters of the production process and localization in hardly accessible places for antimicrobial agents. At the same time, the life of bacteria in the biofilm structure increases the resistance of pathogens and the probability of their survival in adverse conditions. However, the effectiveness of biofilm removal is still not satisfactory, despite the constant improvement of the cleaning and disinfection procedures of industrial surfaces. The methods of biofilm eradication used so far can be divided into three groups (physical, biological, chemical). Numerous in vitro studies indicate that alternatives to commonly used disinfectants may be natural substances such as essential oils that have bactericidal and bacteriostatic activity. The present review will focus on describing biofilm formation and performance. In addition, the paper describes an overview of the methods used to prevent and eradicate biofilms.*

***Keywords:*** *biofilm, production environment, food, eradication.*

## Introduction

Food available on the market should be safe and of a high microbiological purity. Therefore, there is a need for continuous monitoring of production lines and microbiological control of the final product. The proper hygiene of the production environment can be maintained by appropriate methods of cleaning and disinfection. However, the problem of the industry are microorganisms acquiring resistance to disinfectants used in the production facility [1,2]. It turned out that in comparison to free-flowing cells, microorganisms growing in the biofilm structure are nearly 100-1000 times more resistant to toxic substances - disinfectants, surfactants, antibiotics, antiseptics and proof to defense action of the human body cells than those remaining in planktonic form [3]. Previously available antibacterial agents may show lower activity in relation to phenotypically different bacteria in the biofilm structure, because they were selected and implemented based on their high activity against planktonic bacterial populations, according to classic indexes such as a minimal inhibitory concentration (MIC) and a minimum bactericidal concentration (MBC) [4].

In the industry, biofilms are multi-species sets of microorganisms colonizing places that are hard-to-reach for washing agents. The components of biofilms may be

saprophytic and pathogenic bacteria, including: *Salmonella* spp., *Listeria monocytogenes, Escherichia coli, Campylobacter* spp., *Bacillus cereus* or *Staphylococcus aureus* [5].

The qualitative composition of the biofilm varies depending on the raw materials used and the parameters of the production process. At the same time, the bacteria viability in the biofilm structure may increase under cooling and freezing, pasteurization, reduced acidity, high salinity, or in the presence of disinfectants. In order to streamline technological processes and develop effective methods for combating biofilms, it is necessary to recognize its structure, mechanism of creation and functioning [6]. The presented review focuses on bacterial biofilms in food industry production environments, their development and eradication.

## General characteristics of biofilm

### Biofilm formation

The process of creation and maturation of the biofilms depends, inter alia, on the physical and chemical parameters of the environment e. g. availability of the oxygen, osmotic pressure and pH as well as a type of surfaces colonized [7]. Biofilm formation is a multi-stage process and also depends on microorganisms' type and physiochemical properties of colonized materials or a host organism. The adhesion process is modulated by the hydrophobicity of the cell surface, cell wall proteins, extracellular polymers produced by microorganisms, lipopolysaccharides, as well as extracellular structures such as fibriae and flagella [8]. Fibroblasting cells equipped with hydrophobic groups more easily overcome the repulsive forces created between the negatively charged bacterial cell wall and the settled surface negatively charged. This mechanism is important in conditions of stagnation. On the other hand, the bacterial pili and flagella may influence the adhesion and biofilm formation through various mechanisms depending on the type of bacteria. These organelles help the bacteria reach the surface in order to create a new or enlarge the existing biofilm [9,10]. Colonization is also facilitated by the structure of the surface and all its damage and roughness [11]. The mechanism of biofilm formation is not yet well understood, but the following phases can be observed in the biofilm formation process: (i) reversible adhesion of microorganisms, (ii) irreversible adhesion, (iii) biofilm maturation and (iv) biofilm dispersion [12]. The initiating step is the process of adhesion of individual cells to the surface. The first stage of adhesion is reversible and conditioned by many interactions between the bacterial cell wall and the colonized surface. Movement of cells regulates hydrodynamic, gravitational, thermodynamic, electrostatic, and hydrophobic interactions, whose broad spectrum of action allows the cells to approach the surface. It is also determined by overcoming spherical obstacles, Van der Waals forces and temperature [7]. In the stage of irreversible adhesion, as a result of intercellular and environmental interactions, the microbial cells are irreversibly bound to the colonized surface. Then, there are specific chemical interactions that lead to the formation of hydrogen bonds, the formation of vapors and complexes (covalent bonds of the carbon-carbon type). In this phase of biofilm formation, microorganisms start the production of EPS (extracellular polysaccharides) which creates a glycocalyx, allowing free living bacteria to join the newly formed structure [7]. Interestingly, the creation of a specific cluster of one species of microorganisms stimulates the adhesion of others [13].

Irreversible adhesion allows microcolony production and the biofilm maturation. Multiplication of microorganisms and their gradual differentiation and change of expression of some genes take place altogether with the production of flagellin, some

enzymes and toxins inhibition, and the acceleration of anaerobic metabolism. Gene expression is growing enabling bacterial cells to pump out intracellular toxins, as well as antibiotics outside the biofilm (drug effux pumps) [14]. The growth is decreasing, and the metabolism of microorganisms is slowing down. The biofilm formation process is controlled by a *quorum sensing* mechanism (QS) - a unique intercellular communication system that increases the biofilm adaptive abilities [10]. In the last phase of development, the biofilm achieves critical thickness and gradually ceases to maintain the existing form. Bacterial cells regularly detach from the structure and EPS production is stopped to start the process of expansion of new surfaces. The biofilm cell dispersion occurs by separating the newly formed cells from growing cells or the dispersion of biofilm aggregates as a result of flow effects or *quorum sensing*. Dispersed biofilm cells have the ability to retain certain properties of the biofilm, such as sensitivity to antibiotics. Cells dispersed from the biofilm can quickly return to the normal planktonic phenotype [15].

## Biofilm structure and performance

Biofilm can be single- or multi-layered, produced by one species or many species of microorganisms [16]. The construction of biofilm depends on many factors, physical as hydrodynamic conditions, as well as general and specific biological factors. General biological factors, such as growth performance and substrate conversion rates, are the basic factors determining the formation of its structure. Specific strain-dependent factors such as bacterial mobility, cell-to-cell communication, exopolysaccharides or proteins modify them, resulting in further differences between different biofilm systems [17].

Depending on the environment, three basic types of these microbial communities are distinguished: flat (two-dimensional), columns and mushroom or tulip structure [18]. The cells of the microorganisms within the biofilm are connected with each other by EPS, whose components play an important role in the creation and functioning of biofilm, including adhesion, protection and structure. EPS is responsible for the adhesion of planktonic cells to both biotic and abiotic surfaces. Polysaccharides may also provide protection against physical stresses caused by fluid movement, bactericidal agents, immune effectors and predators such as phagocytic cells and amoebae. In addition, polysaccharides provide structure to biofilms, allowing stratification of the bacterial community and the formation of gradients of nutrients and metabolic products [10, 19]. The composition of extracellular polymers of biofilm is very diverse and depends on the bacterial species, the age of the biofilm, and its amount depends on the quantitative and qualitative composition of nutrients. Stimulation of its production is induced by a reduced content of nitrogen and oxygen, potassium and phosphates, with a simultaneous excess of an easily absorbable carbon source [20]. In addition, its production is affected by pH of the substrate, the incubation temperature and the growth phase of microorganisms [20, 21]. In the mature biofilm, rhamnolipids play an important role and influence intercellular and intracellular interactions. They are responsible for maintaining the biofilm architecture, ensuring the flow of nutrients and oxygen within the structure and the outflow of metabolites and toxic substances. In addition, rhamnolipids prevent the colonization of open spaces in the biofilm by external bacteria [22]. Cells located inside the biofilm are less active or remain in the state of anabiosis. However, the top layers removal can cause them to be activated. Biofilm is a dynamic structure with the ability to regulate metabolic processes in more extent than in the population of planktonic cells [23]. The proper performance of the biological membrane provides intercellular signaling

based on the production of signaling molecules that freely diffuse from one cell to another, sensing the concentration of *quorum sensing* cells.

## *Quorum sensing*

*Quorum sensing* is defined as a way of a communicating of the cells enabling the determination of their numbers in the biofilm. QS generates, releases, and recognizes signaling molecules, allowing cells to regulate the expression of specific genes in response to changes in cell population density [24, 25]. The bacteria communication is based on the production of signal molecules - autoinducers and their recognition by specific receptor proteins. Achieving the quorum, and thus the specific concentration of the autoinducer, is a signal to activate the expression of genes that control vital microbial life processes and maintain physiological and metabolic processes relevant to the entire population. In this way, sporulation, cell differentiation, biosynthesis of secondary metabolites, plasmid transfer, virulence, bioluminescence, DNA replication, production of enzymes and toxins, and resistance to antibiotics are regulated. This mechanism coordinates physiological and metabolic processes within biofilm. Communication of microorganisms can occur between cells of one or different species, and even between cells of prokaryotic and eukaryotic organisms [26]. The chemical structure of signaling molecules, the mechanism of their action and genes controlling the phenomenon of cell communication differ between species. Gram-positive and Gram-negative bacteria have developed different signal-transmission systems [27]. Gram-negative bacteria use acylated homoserine lactones (AHLs) as autoinducers, while Gram-positive bacteria use specific oligopeptides [28].

Gram-negative microorganisms such as *Pseudomonas aeruginosa, Aeromonas hydrophila, Burkholderia cepacia, Yersinia pseudotuberculosis, Serratia liquefaciens, Serratia marcescens* communicate via AHL signaling molecules (N-acylthomoserine lactone), which have identical polar head groups but may differ in hydrophobic acyl groups by length, oxidation or desaturation. As a result, each bacteria species has its own language not understandable for other microorganisms [29]. In addition, signal molecules of Gram-negative bacteria, unlike Gram-positive ones, diffuse through the cytoplasmic membrane [30]. Binding the autoinducer molecule to the LuxR protein leads to the activation of transcription of target genes that allow cells to interact in the biofilm structure. It also turned out that the ability to produce AHL autoinducers can be transmitted by horizontal gene transfer to other cells without the ability to synthesize autoinducers themselves [10].

Gram-positive bacteria developed signaling oligopeptides in a two-factor system of detection and response to the presence of an autoinducer [31]. Oligopeptides are detected by membrane-spanning kinases or by cytoplasmic receptors.

There are nonspecific autoinducers for communication between microorganisms belonging to different species. These are AI-2 molecules (autoinducer-2), involved in the regulation of threshold sensitivity mechanisms in both Gram-positive and Gram-negative bacteria *Bacillus, Clostridium, Enterococcus, Escherichia, Haemophilus, Helicobacter, Klebsiella, Lactobacillus, Leuconostoc, Listeria, Mycobacterium, Proteus, Salmonella, Shigella, Staphylococcus, Streptococcus, Vibrio, Yersinia* [10, 32, 33]. Virulence, toxin production, bioluminescence, conveying plasmid transfer, biosynthesis of antibiotics, and aggregation and differentiation of biofim are regulated in this way [31].

## Biofilm in food industry

Biofilms create difficulties in maintaining production hygiene, and bacterial biofilms are reservoirs of cells that contaminate products. Biofilms can also cause technological problems, such as hindering the heat flow in heat exchangers and extractor, increasing the abiotic surfaces microbiological corrosion leading to energy and production losses, and significantly reducing the effectiveness of washing and disinfection [34, 35].

If biofilms developed on food contact surfaces are not completely removed, cross-contamination may occur, which is dangerous especially in the dairy and meat industry. Modern food processing favors the creation of biofilms and the selection of bacteria adhering to abiotic surfaces [36]. Complexity of technological lines, mass production, long production cycles and vast surfaces food contact create environment for unperturbed formation and development of bacterial biofilm [36, 37].

Literature data indicate the prevalence of biofilm formation of pathogenic bacteria on food producing surfaces [38]. Among bacteria commonly found in the meat processing environment are: *Pseudomonas* spp., *Brochothrix thermosphacta, Lactobacillus* spp., *E. coli, Acinetobacter* spp., *Moraxella* spp., *Leuconostac* spp., *Enterococcus* spp. and pathogens *S. enterica, L. monocytogenes* [39]. In the dairy industry, usually the internal surfaces of the pasteurization lines, are colonized by biofilms of pathogenic bacteria: *E. coli, L. monocytogenes, Yersinia enterocolitica, S. aureus, Salmonella* spp., *B. cereus* [40]. In the fish industry, biofilms form pathogenic bacteria are mainly composed of *Vibrio* spp., *Aeromonas hydrophila, Salmonella* spp. and *L. monocytogenes* [41].

In fresh fruits and vegetables processing biofilms are formed by *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp., *L. monocytogenes, Campylobacter* spp. [42,43]. In the juice industry the problem is a biofilm created by the acidothermophilic bacteria *Alicyclobacillus acidoterrestris*. The cells and spores of these bacteria form a biofilm on the surface of stainless steel, PVC and nylon, difficult to be removed even by solutions of quaternary ammonium salts [44]. It should be emphasized that the formation of biofilms under certain conditions is desirable. The production of fermented meat utilizes technologically useful bacteria *Staphylococcus* and *Lactobacillus*, stimulating meat fermentation, providing protection against putrefactive bacteria biofilms and pathogens [39].

Surfaces of technological lines in the food industry are the environment particularly suitable for the development of biofilms due to the access of nutrients in the long periods of the production cycle. Product residues on the surfaces act as a bacterial biofilm conditioning agent. It was found that biofilms formed by *L. monocytogenes, Salmonella* spp. and *Y. enterocolitica* on the surfaces of devices used to grind meat contaminated with residual raw material are removed with much lower efficiency than biofilms formed on the clean surfaces of the same devices [45].

The presence of bacterial biofilms on industrial surfaces poses a risk of cross-contamination in the consumers' homes. Food poisoning outbreaks caused by cross-contamination in private homes were reported three times more than those having a source in mass catering [46].

## Biofilm prevention and eradication

Although the procedures for cleaning and disinfecting industrial surfaces are being developed and constantly improved, the effectiveness of biofilm removal is still unsatisfactory. The industry uses many disinfectants like chlorine, iodine, chloramides, hydrogen peroxide, ozone, peracetic acid or quaternary ammonium salts, that may create

bacteria resistance. Therefore, there is a need to search for new substances that effectively destroy bacterial biofilms. Numerous *in vitro* studies indicate that alternatives to commonly used disinfectants may be natural substances such as plant extracts, essential oils (EO), and even hydrosols with bactericidal and bacteriostatic activity. These compounds easily penetrate through the wall and cellular membrane of microorganisms causing disruption of the integrity of these structures eventually leading to the lysis of bacterial cells. The cytotoxic effects of EO, notably some of the terpenes, have been observed *in vitro* in many types of bacteria, in particular Gram-positive bacteria, to a lesser extent Gram-negative, which is probably associated with differences in the structure of their cell wall. The advantage of natural substances is low or no toxicity, full degradability and acceptance of consumers who expect a natural way to produce food. In addition, these substances are characterized by a relatively high antimicrobial activity and the lack of resistance of the bacteria [47].

Inhibition of biofilm formation is believed to be related to the presence of phenols (thymol, carvacrol), e.g. the compounds of essential oils of oregano and thyme. The studies using carvacrol were carried out in 2001 against the biofilm created by the bacteria *L. monocytogenes, S. enterica, S. aureus* and yeast *Saccharomyces cerevisiae* on the surface of stainless steel. It turned out that the effectiveness of carvacrol was comparable to that of commercial disinfectants containing hydrogen peroxide and peracetic acid [48]. The practical use of essential oils or their components and plant extracts for the eradication of biofilms in food-producing factories is, however, limited due to their intense odor. The use of these substances in concentrations above 1% may affect the organoleptic characteristics of the products. Therefore, the solution may be mixtures of synthetic disinfectants with substances of natural origin. Studies of such mixtures indicate that even mild micellar surfactants in combination with eugenol and carvacrol inhibited the growth of biofilm *L. monocytogenes* and *E. coli* O157:H7 [49].

The methods of biofilm eradication used so far can be divided into physical, biological and chemical ones. The basic physical method with high effectiveness is the mechanical destruction of the biofilm structure using scrubbing or scraping. However, it cannot be used in hard-to-reach places, reducing the universality of the method. Another physical method is the operation of high and low temperature. The use of temperature above 95°C for 100 min usually completely destroys biofilms. Similar effect brings freezing and thawing the biofilm structure three times at -12°C [50]. Attempts are made to eliminate biofilms by using ultrasonic waves [51] and an electric field. *In vitro* studies indicate that 150 pulses are enough to remove 80% or even 100% of the biofilm [52]. There are high expectations for methods using cold plasma as a mixture of free radicals, ions, excited molecules and UV radiation that can cause the disorganization of the cell membrane, destruction of intracellular proteins and DNA structure. Because cold plasma is essentially dry, non-thermal and chemical-free technology, it offers some potential advantages over conventional washing systems. In addition to economic aspects, cold plasma does not require the use of conventional chemicals and leaves no residues, which makes this method interesting for the food industry and the medical environment [53]. An interesting alternative is also the photodynamic therapy, the negative effect of which is reduced by the combination with silver nanoparticles [54].

Among the biological methods, the high activity of bacteriophages in relation to Gram-positive and Gram-negative bacteria was confirmed. Bacteriophages, due to their high specificity, make a significant promise in combating biofilms. Disinfectants based on bacteriophages meet all the requirements for effectiveness and safety. However, such

applications are still evolving, and large-scale applications are still in the research phase [55, 56].

The fight against microbes can be facilitated by the hydrolytic enzymes degrading AHL signaling molecules. These enzymes interfere with transcription, followed by the formation of biofilm. They belong to three classes: AHL lactonase, AHL acylase and oxidoreductases [57]. Another strategy for biological eradication is the use of enzymes directed towards the polysaccharide matrix (matrix targeting enzymes). Interference with the structure or degradation of the extracellular polymer matrix of the biofilm can effectively weaken it or lead to its dissipation. However, the efficiency of enzymes on the matrix has not yet been confirmed in *in vivo* tests. There are many limitations in this type of approach, one of which is the risk of inflammatory and allergic reactions [50, 58].

The activity of many available antimicrobial compounds in relation to the biofilm is significantly reduced compared to the activity of these substances against planktonic cells. This is mainly due to the layered structure of the biofilm and the presence of the polysaccharide matrix, which makes it difficult to penetrate the compounds into the structure. In addition, there are changes in gene expression and metabolism of microbial cells located in the deeper layers of the biofilm, leading to reduced sensitivity to many antimicrobial substances [59].

The chemical methods used are mainly based on the use of oxidizing substances, e.g. chlorine and its compounds, which cause degradation of the spatial structure. The formaldehyde and quaternary ammonium salts or surfactants are also effective against the biofilm, the mechanism of which is to disrupt the integrity of the structure. However, the use of the above-mentioned chemical compounds is limited to surface disinfection [29].

## Biofilm answer to antimicrobial substances

Biofilm formation with a multidimensional and dynamic nature allows single-cell organisms to adopt a temporary multicellular life style that facilitates survival in adverse conditions. The transition from planktonic growth to biofilm occurs in response to environmental changes and includes many regulatory networks that transmit appropriate signals to start expressing relevant genes, mediating spatial and temporal reorganization of the bacterial cell [60]. The main reason for the biofilm resistance is surrounding the cells of biofilm with sticky polymer (EPS), the amount of which increases as the biofilm matures. The living conditions inside the biofilm will also affect the potency of antibacterial substances. The oxygen content is limited and anaerobic conditions prevail in the deeper layers. Therefore, some of the cells become anabiotic, and metabolism and multiplication are ceased. Because some antibacterial agents act only on metabolically active cells, the transition to the stationary phase of the part of cells in the inner zone of biofilm may be associated with a significant reduction of their sensitivity to these substances. In mature biofilm, genes responsible for the synthesis of enzymes decomposing antibacterial substances are also activated. It is also believed that biosurfactants produced by some bacteria (e.g. rhamnolipids) contribute to the increase of bioimmune resistance to phagocytosis and actively participate in disease infections [10, 61].

## Conclusion

Bacterial biofilm is a complex structure colonizing both biotic and abiotic surfaces. In industrial environments biofilm development serves a serious threat to product microbial quality and a consumer health. Among variety of methods used to control and eradication of biofilm none is fully effective. The implementation of nanotechnology techniques or

natural origin substances for bacterial biofilm control may be an alternative for synthetic disinfectants evolving bacterial resistance to antimicrobials.

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