Biofilm

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The ability of biofilm formation is an ancient property of bacteria (and other prokaryotes) that represents, from the evolution point of view, the survival strategy in variable and often highly unfavourable conditions of the environment. Contrary to characteristics that bacteria show during their growth in the media abounding with nutritive substances, bacteria in biofilms show different properties in terms of genes expression and growth characteristics. Due to the presence of these differences, bacteria in biofilms show an increased resistance to antibiotics and disinfectants and that is why it is almost impossible today to treat infections caused by biofilms with conventional antibiotics, just as it is not possible to control bacteria in the hospital environment and places of food production using disinfectants in recommended concentrations. In medical and industrial microbiology, the concept of biofilms formed by bacteria was accepted during the 1990s and it is currently one of the greatest challenges in the field of protection of human health and production of healthy safe food. This mini-review will discuss stages in biofilm development, quorum sensing, significance of extracellular polymeric substance, bacteria resistance mechanisms in biofilm, significance of biofilm in food industry, as well as the basic methods of static biofilms testing.

Keywords biofilm formation; biofilm resistance; biofilm in food industry

1. History of biofilms

Microbial communities attached to surfaces (biofilms) had been observed long before people had the tools to study them in detail. From a historical point of view, the discovery of microbial biofilms can be attributed to Antonie van Leeuwenhoek, who first observed microorganisms on the plaque on his own teeth. After van Leeuwenhoek’s discovery, the research in this area continued in the thirties of the XIX century [1].

In these early studies, the phenomenon of adherence of bacteria was investigated by Henrici, Windogradsky, Cholodny, and Conn. An important observation made by these scientists was that bacteria that grew attached to the surface express different phenotypic characters from planktonic counterparts [2]. Within this period, the largest scientific contribution in this area was made by ZoBell. ZoBell and Allen (1935) reported the first apparatus specifically designed to examine bacterial attachment to surfaces. It was a carrier that held 16 glass slides and was designed to be lowered into the ocean where marine microbes could attach to the glass. Using this apparatus, they found a greater diversity of bacteria in the biofilm “lawn” on the slide than the one that could be cultured from the sea water [3].

Extensive research in this field started only in the late 1960's, or early 1970's. In 1969, Jones et al used for the first time the transmission electron microscope to study biofilm created on filter irrigation plants, while at the same time, using a special ruthenium-red staining, these scientists showed that the matrix in which the bacteria were immersed was of polysaccharide nature [4].

The turning point in the study of biofilm formation was in the year 1978 when Costerton et al. stated a theory of biofilm development. According to their theory, the majority of bacteria grow in matrix enclosed biofilms adhered to surfaces in all nutrient-sufficient aquatic ecosystems and that these sessile bacterial cells differ profoundly from their planktonic counterpart [1].

The term “biofilm” had been used colloquially among researchers for some years before it was considered a term acceptable for use in publications. The earliest use of “biofilm” in publication is in the Swedish journal Vatten: Harremoës, P. 1977. “Half-order reactions in biofilm and filter kinetics,” Vatten, 33, 122-143 [5]. Even after so many years of research, there are still many unanswered questions about the formation, function, maturation, and cell death during biofilm development.

2. Biofilm definition

In nature, microorganisms can exist as planktonic organisms - individual cells freely suspended in a liquid medium, or as a sessile community - biofilm. In the past two decades, the definition of a biofilm has been constantly changing as each new study builds on the existing knowledge on formation, structure, maturation, and resistance of biofilms. Consolidating the knowledge of the known characteristics, and the newly discovered physiological characteristics, biofilm is now defined as “structural communities of microorganisms that are irreversibly attached to a surface or interface, embedded in an extracellular matrix of polymeric substances which these cells have produced”. Bacteria in biofilms exhibit an altered phenotype with respect to the growth rate and gene transcription in relation to planktonic cells [6, 7].
3. Biofilm formation

With the introduction of new microscopic and molecular methods, the researchers have revealed that biofilms are not simple bacterial layers on different surfaces but rather biological systems with high organization level, where bacteria are developing coordinated and functional communities [8]. Biofilm formation is a complex process that is carried out in several stages: adhesion stage, aggregation stage, maturation stage, mature biofilm stage, and dispersion stage.

Some authors consider the surface conditioning as the first step in biofilm formation, although the conditioning represents the interaction of the surface and its environment [9, 10]. In natural environment, microorganisms do not adhere to the substrate themselves but they rather stick onto this so-called conditioned layer, which is known to be formed on most substrates as the result of chemical surface modification [9]. In this way, bacteria adhesion will depend on compatibility of macromolecules of the conditioned surface with surface properties of bacteria, which can either facilitate or reduce bacteria adhesion onto the substrate. The role of the conditioned layer is in its ability to modify the physical and chemical properties of substrate, and to serve as the source of nutritive substances and important microelements.

4. Adhesion

Adhesion of microorganisms onto the substrate plays the key role in biofilm formation. The process of adhesion onto a certain surface is carried out through two stages: primary adhesion (the stage of reversible binding) and secondary adhesion (the stage of irreversible binding). The first step in biofilm formation occurs when microorganisms encounter the surface, which enables the primary bacteria adhesion. Bacterial cells could be moved passively (due to the effects of hydro-dynamics, Brown’s motion, or sedimentation) [11] or actively (due to locomotor organs, or taxis). When bacteria come close enough to the surface, the adherence will depend on the net sum of the attraction and repulsion forces created between two surfaces.

Attraction and repulsion forces involved in the process of adhesion of bacterial cells to the surface include Van der Waals’ forces at the distance of 50 nm, Van der Waals’ and electrostatic forces at the distance of 20 nm when the bond between bacterial cells and substrate is reversible. At the distance of 1.5 nm, there are also ion bonds and hydrophobic interactions [12, 13]. Once the attraction forces get stronger than repulsion forces, the binding to the substrate becomes irreversible. The transition from reversible into irreversible adhesion also involves different short-range forces such as
covalent, ion, and hydrogen bonds and hydrophobic interactions. Due to small dimensions of bacterial cells, the gravity (i.e. sedimentation) is negligibly small, and the highest significance in the contact of bacteria with the surface is attributed primarily to the bacteria mobility.

Diverse surface structures, such as fimbriae, flagella and pili, which play the key role in the adhesion phenomenon, can be found on the surface of bacterial cells. Fimbriae are amyloid cell-surface proteins, and are involved in adhesion to surfaces, cell aggregation, environmental persistence and biofilm development. Flagella participate in the initial binding of bacterial cells with the surface by possessing numerous ion groups that are covalently bonded with surface molecules around the cell wall leading to a strong electrostatic interaction of bacteria with the surface, while pili enable twitching motion generated by pili extension and retraction. In the temporal function, the bond between bacteria and substrate is stronger, and it turns binding into irreversible process. Strengthening of the bond between bacteria and substrate is the result of production of extracellular polymeric substance (EPS), or biofilm matrix.

5. Formation of microcolonies and biofilm maturation

Aggregation is the second stage of biofilm development and it is the result of simultaneous accumulation, growth, and multiplication of microorganisms. The cell division initiates the “quorum sensing” (QS) molecules and further EPS production. In addition to enabling aggregation of bacterial cells by forming microcolonies, biofilm matrix contributes to further adhesion of biofilm to the surface on which it is formed [14].

Further biofilm development is the result of adherence of new planktonic cells in combination with continuous growth of already bonded cells and EPS production. Within the formed microcolonies, bacteria are bonded with intercellular bonds, surrounded with EPS that binds them together. The growth potential of one bacterial biofilm is limited with availability of nutrients, capacity of their perfusion to the cells and elimination of metabolic products. Other factors that affect biofilm maturation include pH, oxygen perfusion, sources of carbon and osmolarity [10].

Biofilm maturation changes the conditions within the micro-environment that surrounds the bacteria in terms of cell population density, pH, presence of nutrients and oxygen, while the difference in metabolic and reproductive cell functionality arises as the consequence of heterogeneity of the environment [14].

During the initial colonisation, oxygen is not a limiting factor, but the position of individual cells in a multi-layered robust biofilm is determined by their physiological status. The cells in the surface biofilm zones get nutrients and oxygen more easily and they easily release waste metabolic products. Physiological status of these cells is very similar to the one of planktonic cells. Bacteria in the zone with enervated oxygen inflow continue the respiration whereby nitrate and other inorganic compounds serve as electron acceptors. Transport of nutrients to the cells and discharge of waste metabolic product is enabled via the interstitial water channels.

The biofilm development process is quite slow, and biofilm can be evaluated as mature after several days [15]. Fully mature biofilm consists of bacterial cells, EPS, and interstitial water channels that enable the exchange of nutrients and elimination of waste metabolic products [16].

6. Dispersion

Dispersion is the last stage of biofilm development that is characterised by deadhesion of microorganisms from the biofilm structure. Dispersion arises as the response to the changed environmental conditions, irrespective if they are caused by the lack of nutrients or by other unfavourable impacts. The dispersion mechanism can be active (implies the mechanisms initiated by the bacterial cells themselves) or passive (refers to dispersion of bacteria mediated by shear forces, abrasion, human factors, predation over bacteria in biofilm) [14].

Deadhesion of bacteria from the biofilm can be carried out via erosion, sloughing, seeding, and abrasion [14, 17]. Erosion is a continuous detachment of individual cells or small biofilm fragments that occurs to a very small extent during the process of biofilm formation. The biofilm erosion rate grows with the increase of matrix thickness and during the increased action of shear forces. Biofilm sloughing implies the abruption of massive pieces of biofilm, usually during the late stages of its formation. It is believed that it arises most often as the consequence of the lack of nutrients and oxygen. Generally speaking, cohesiveness is under a strong impact of ECM structure and composition that depends on the growth and development stage of the biofilm.

Seeding via dispersal implies the release of a large number of individual cells or smaller aggregates from the biofilm inside. Erosion and sloughing can be both passive and active processes, while seeding is always an active process [14]. Abrasion is also mentioned as the mechanism of biofilm dispersion. It implies the loss of biofilm initiated by collisions of particles from the liquid surrounding the biofilm [17].

Just as it is the case with all process involved in biofilm formation, the processes involved in its dispersion are also very complex. These processes include a wide spectrum of biochemical, ecological, and physical triggers as well as the activation of signalling transduction paths. Production of EPS-dissolving enzymes, lithic activation of bacteriophage, expression of phosphodiesterase and QS signals are most frequently mentioned biochemical factors involved in the process of bacterial deadhesion. Ecological factors that are included in deadhesion process imply mainly the limitations
to nutrients, accumulation of metabolites, changes in osmolarity and a high growth rate. The most frequent physical factor is the presence of shear forces caused by continuous flow of liquid [18]. Biofilm growth and deadhesion are mutually dependent processes, and deadhesion rate increases with biofilm maturation. In essence, the dispersion of microorganisms from biofilm can be defined as a kind of adaptive response to the conditions present in the environment in which biofilm is developing. As the adaptive response to the starvation conditions, *Pseudomonas aeruginosa* cells produce the alginate lyase enzyme that dissolves alginate, namely the biofilm polysaccharide [19]. A similar enzyme, dispersin B, has been detected in *Staphylococcus epidermidis* specie [20]. This enables the use of matrix as the source of nutrients aimed at avoiding the starvation, while, on the other hand, matrix dissolving provides for dispersion of microorganisms enabling them to search for niches with more available nutrients, which is of the key importance for a long-term survival.

7. Extracellular polymeric substance (EPS)

The production of extracellular polymeric substance or biofilm matrix is the main precondition for biofilm formation [21-23]. Biofilm matrix is highly hydrated substance, which consists of water, exopolymer, microorganism, and products of their activities. Biofilm matrix exopolymers are usually composed of polysaccharides (40-95%), proteins (1-60%), nucleic acids (1-10%), and lipids (1-40%). The composition of EPS depends on the present microorganisms, temperature, and availability of nutrients [24].

EPS is the basis of three-dimensional biofilm matrix structure and it is responsible for adhesion of biofilm to surfaces as well as for its consolidation (cohesion). Although there is a large number of exopolymers and their functions as integral parts of EPS, they have not been tested in details yet. The set functions show a wide spectrum of advantages of life in biofilm (Table 1).

<table>
<thead>
<tr>
<th>Function</th>
<th>Relevance for biofilms</th>
<th>EPS components involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion</td>
<td>Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces</td>
<td>Polysaccharides, proteins, DNA and amphiphilic molecules</td>
</tr>
<tr>
<td>Aggregation of bacterial cells</td>
<td>Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition</td>
<td>Polysaccharides, proteins and DNA</td>
</tr>
<tr>
<td>Cohesion of biofilms</td>
<td>Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication</td>
<td>Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and DNA</td>
</tr>
<tr>
<td>Retention of water</td>
<td>Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments</td>
<td>Hydrophilic polysaccharides and possibly, proteins</td>
</tr>
<tr>
<td>Protective barrier</td>
<td>Confers resistance to non-specific and specific host defences during infection, and confers tolerance to various antimicrobial agents</td>
<td>Polysaccharides and proteins</td>
</tr>
<tr>
<td>Sorption of organic compounds</td>
<td>Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)</td>
<td>Charged or hydrophobic polysaccharides and proteins</td>
</tr>
<tr>
<td>Sorption of inorganic ions</td>
<td>Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)</td>
<td>Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate</td>
</tr>
<tr>
<td>Enzymatic activity</td>
<td>Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms</td>
<td>Proteins</td>
</tr>
<tr>
<td>Nutrient source</td>
<td>Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community</td>
<td>Potentially all EPS components</td>
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autoinducer-2 (AI-2) in Gram-negative and Gram-positive bacteria, and autoinducer-3/epinephrine/norepinephrine (AI-3/epi/norepi) in some species of enterobacteria [27]. Quorum sensing is considered the key process in regulation of expression of genes responsible for different physiological activities such as competition, symbiosis, motility, sporulation, bioluminescence, production of antimicrobial peptides, virulence regulation, as well as some of the genes that are responsible for biofilm formation regulated by “quorum sensing” system [27, 28]. Since “quorum sensing“ depends on population density it does not occur in the initial but rather in later stages of biofilm formation.

In the previous period, it was believed that intercellular communication was present only in several species (Vibrio fisheri, Vibrio harveyi, Enterococcus faecalis, Myxococcus xanthus, and Streptomyces spp.). The studies in the field of intercellular communication found the presence of a large number of signalling molecules. Until now, four classes of these molecules have been described in detail: N – Acyl – Homoserine Lactones (AHL) or autoinducer-1 (AI-1) in Gram-negative bacteria, autoinducer polypeptides (AI-P) in Gram-positive bacteria, furanosil borate diester or these molecules have been described in detail: N – Acyl – Homoserine Lactones (AHL) or autoinducer-1 (AI-1) in Gram-negative bacteria, autoinducer polypeptides (AI-P) in Gram-positive bacteria, furanosil borate diester or

**8. Quorum sensing**

Quorum sensing represents an intercellular communication between bacteria that is carried out through production of the signalling molecules marked as autoinducers (AI) [26, 27]. In low population density, the production of signalling molecules is at the basal level; as the population density increases the production of signalling molecules grows. Since signalling molecules pass through a membrane of bacterial cells, their concentration in the environment is almost equal to the concentration in the cell. Upon reaching the critical concentration, the signalling molecules return into the cell via diffusion or binding for specific receptors, which initiates a range of cascade reactions that enable coordinated expression or repression of appropriate sets of genes. Since this phenomenon is conditioned with the population density, namely it occurs once the appropriate population density (quorum) is reached, it was called quorum sensing [26].

Resistance to antimicrobial agents implies the possibility for bacteria to avoid lethal effect of the applied antimicrobial agent. Bacteria may accomplish avoiding the lethal effect in several ways: by its reduced adoption from the external environment via modification of the target spot on the receptor or reduction of membrane permeability, increasing of its excretion from the cell (active efflux), as well as via inactivation and hydrolysis of antimicrobial agent, which prevents its activation in the cell [29].

**9. Resistance of bacteria in biofilm**

Microorganisms enclosed into the biofilm matrix show resistance to antimicrobial agents compared to planktonic cultures of the same microorganism [30]. The researches in this field show that resistance of microorganisms enclosed into the biofilm formation may be up to 1,000 times higher [31].

The conventional resistance mechanisms contribute to the overall resistance of bacteria within the biofilm to antimicrobial agents, and so do the mechanisms reserved exclusively for the growth of bacteria inside the biofilm (Figure 2) mediated by extracellular matrix, adaptive response to stress, changes in conditions of micro-environment, occurrence of persister cells and genetic transfer between the cells [32-34].

The extracellular biofilm matrix represents the barrier for antimicrobial agents, disables their transport to bacteria via the interaction with antimicrobial agents and their inactivation, or production of enzymes that dissolve antimicrobial agents of larger molecular mass. Diffusion of antimicrobial agent through the biofilm matrix towards deeper layers causes reduction of antimicrobial agent concentration so that only surface biofilm bacteria are exposed to lethal
concentrations. The extracellular biofilm matrix acts also as a dilution gradient as it slows down the penetration of antimicrobial substances, which provides additional time for cells to transfer new genes for resistance before they are caught by antimicrobial agent [35].

Bacteria are capable of developing diverse mechanisms as adaptive responses to the environmental conditions, which enables them to adapt to new environmental conditions such as sudden change of temperature, oxidative stress, low water activity, DNA damages, and starvation. A large number of adaptive responses of this type has been characterised in details in bacteria suspended in liquid media, and it is most probable that such adaptations are present in bacteria in biofilm. Catalase production in Pseudomonas aeruginosa via the activation of Kat B inducible gene in the response to treatment with 50 mM of hydrogen peroxide is one of simple examples of adaptive responses caused by changes of the environmental conditions. The application of the same treatment to bacteria population suspended in the liquid media does not cause the activation of the above-mentioned gene [36].

The development of biofilm changes the conditions of the microenvironment that surrounds bacteria in terms of availability of nutrients, oxygen, and waste metabolic products [31, 37]. It is believed that the occurrence of metabolically heterogeneous populations within the biofilm with aerobically growing cells on the surface and metabolically inactive cells within the biofilm that are deprived of nutrients and oxygen can play the role in enhancing of resistance to antimicrobial agents [38, 39]. The above-mentioned factors affect the speed of multiplying of bacteria cells favouring the transfer of bacteria into a stationary stage where they are less sensitive to the effects of antimicrobial substances [40]. Most authors are of the opinion that limiting of nutrients in the biofilm zones deprived of oxygen is the cause for the occurrence of resistance rather than the extracellular matrix as physical barrier [41].

In addition to the enhanced resistance to a wide spectrum of antimicrobial agents, biofilm bacteria are becoming even a larger problem due to the occurrence of persister cells representing a small part of biofilm population, which remains capable of living despite a long-term exposure to high dosages of antimicrobial agent. Since the period when they were described for the first time (1944) [42], the mechanisms responsible for their emerging and existence has remained unknown. It is assumed that the explanation can be found in the dormant nature of persister. Slowing down its metabolism persisters can avoid all these damages, which makes them insensitive to antimicrobial substances. Testing the effects of antibiotics on bacteria with different availability of nutrients and growth rate, Roberts and Stewart (2004) found that availability of nutrients and growth rate may affect the resistance to antibiotics [40]. Aridi et al. 2003 [43] came to similar findings by determining the existence of correlation between the growth rate of bacteria and sensitivity to antibiotics.

The death of cells within the biofilm is considered important in the development of resistance of bacteria enclosed into the biofilm. Dead cells may act as the source of nutrients, and they can also function as dilution gradients of antimicrobial agent per cell [38, 44]. Cell death can cause the increase of biofilm attrition rate, which contributes to its dispersal [45]. Biofilm of microorganisms is a perfect milieu for horizontal transfer of genetic material and emerging of pathogens with new virulence factors, resistance, and enhanced capacity of survival in the environment. Horizontal gene transfer can arise via transformation, transduction, or conjugation [46].

Despite extensive studies that have been conducted within the last decade, it is still not possible to determine precisely which of the above-mentioned mechanisms is responsible for the occurrence of resistance of bacteria within the biofilm. The most widely spread explanation of the resistance phenomenon is the occurrence of target modifications via mutations or enzyme changes, or complete modification of the target spot [47].

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**Fig. 2** Hypothesized biofilm resistance mechanisms [36]. 1) The antibiotic penetrates slowly or incompletely; 2) a concentration gradient of a metabolic substrate or product leads to zones of slow or non-growing bacteria (shaded cells); 3) an adaptive stress response is expressed by some of the cells (marked cells); 4) a small fraction of the cells differentiate into a highly protected persister state (dark cells).
The resistance of bacteria in the biofilm is most probably the consequence of the activity of several rather than a single mechanism. Regrettably, the combination of different mechanisms of resistance can often display additive effect leading to the emerging of multiple or even “pandrug” resistance (resistance to all currently available antimicrobial medications), as it is the case with some *Pseudomonas aeruginosa* species.

### 10. Biofilm in food industry

A large number of studies confirmed that biofilms formed by bacteria on different surfaces in food industry plants make a long-term source of contamination of foodstuffs, not only with bacteria causing their spoiling but also with food-borne species such as *Salmonella* spp., *Campylobacter* spp., *Escherichia coli* and *Listeria monocytogenes*. The data that transmission of food-borne pathogens is more and more frequently carried out via foodstuffs of non-animal origin is deeply concerning. Within the last five years, there have been 37 registered epidemics of salmonellosis caused by food of non-animal origin such as fresh salad, tomato, germinated seeds, fruit juices, melons, and nuts. In the largest number of cases, the contamination of plant products occurs by direct contamination with animal faeces, or indirectly by contaminated water (irrigation), equipment [48], and land/soil [49]. The latest studies indicate the facts that have not been known until now in the field of natural distribution of this specie of bacteria. Namely, in addition of contamination of surfaces of fruit and vegetables with external sources, the capacity of *Salmonella* to infect the plants, with intracellular position and active multiplication and with consequential pathogenic effects in all stages of plant development has been proven. The studies in this field indicate that *Salmonella* may infect intracellularly lettuce, tomato, different germs, etc. causing the cascade of immune response and pathological changes [49]. The application of high resolution microscopy technique proved the capacity of food-borne pathogens to form stable biofilms on plant roots and leaves [50].

It is known that some food-borne pathogens in food production plants may exist throughout several months, even years. These strains are known as “house strains”, and the assumption is that the existence of such strains is enabled due to their ability to form biofilms [51]. Several studies confirmed the ability of adherence and biofilm formation of food-borne pathogens on different types of materials that are usually used in food industry [52-55]. Biofilms of food-borne pathogens are found on conveyor belts, cutting and packing machines and other surfaces that get in contact with food. Bacteria in biofilms survive for months and even years and that is why the equipment in food industry represents the potential source of food contamination. Having in mind the consequent effect that biofilm formation may have in food industry, the biofilm control in food industry plants becomes the imperative in production of healthy safe food.

Technological procedures and handling during food processing, sale and preparation of food have increased the need for sanitary procedures and hygiene conditions in both food and feed industry. The efficient sanitation programme should represent the basis for the system of health safety assurance. The application of such programmes is necessary to ensure health safety and quality of products. The application of disinfectants in food industry depends on their efficiency, toxicity, easiness of removal and consequential impact on sensory quality of the final product. The implementation of conventional sanitation methods usually includes the use of aggressive compounds such as chlorine, peroxides, and quaternary ammonium compounds. In addition to the possible residual effect, which may be detrimental to human health, these compounds may cause the reduction of sensitivity of certain types of microorganisms that remain on surfaces causing constant contamination. The efficiency of disinfectants and recommended concentration are the results of tests conducted on broth bacteria cultures. While their application on broth cultures is efficient up to 100% [56-58], the application of the same concentrations on bacteria in biofilms shows inefficiency so that their elimination requires much larger concentrations.

The trend of production and use of healthy safe food without synthetic chemical compounds is becoming more and more emphasised, irrespective if these compounds reached food residually, as the consequence of inadequate sanitation during the technological production procedure, or in the form of preservers and additives. The consumers increasingly require the use of natural products, the so-called “green chemicals” [59] and that is why new control strategies are being constantly developed aiming at finding the potential biological solutions that include antimicrobial compounds of plant origin [60], enzymes, phage, inter-species competitors, or antimicrobial compounds produced by microorganisms [61].

### 11. Basic methods for biofilm testing in static systems

Several procedures have been described as those used to study biofilms in static systems. Their shared characteristic is performing simplicity since they imply the implementation of equipment and accessories that are usually available in most laboratories. Static systems are applicable in testing of adhesion of microorganisms onto the substrate during early stages of biofilm formation and intercellular signalisation responsible for transition into the biofilm phenotype. The main shortcoming of static systems is the limitation in quantity of available nutrients and biofilm aeration, which affects the process of its maturing and formation of biofilm structure typical for systems with continuous flow of liquid [62].
The basic protocols for biofilm testing in static systems include:

1. Microtiter plate biofilm assay;
2. Air-liquid interface assay (ALI);
3. Colony-based biofilm system.

11.1. Microtiter plate biofilm assay

Microtiter plate biofilm assay has been widely used since the mid 1990s, while different modifications of this test originate from the basic protocol that has been described by Christensen et al. (1985) [63]. The test principle is as follows: bacteria culture is inoculated into microtiter plate wells and incubated for the desired period of time at the selected temperature in the substrate that enables bacteria to grow. Upon the expiry of the incubation period, the suspended bacteria are eliminated by flushing while bacteria attached to the surface are painted with crystal-violet solution, which visualises their binding for the substrate. Dissolution of the bonded crystal violet is achieved by adding the solvent (DMSO, ethanol/acetone (80%/20%), 95% ethanol, 30% of acetic acid, 33% of glacial acetic acid), and the quantity of bonded paint is determined spectrophotometrically. The advantage of this method is the possibility of simultaneous testing of a larger number of isolates, which makes it suitable as a screening method that precedes more complicated procedures.

11.2. Air-liquid interface assay (ALI)

This test enables microscopic analysis of biofilms that are formed on a border surface between liquid and air [64]. The simplest way to conduct the test is to set plates (with 24 or less wells) into oblique position (under the angle of 30° to 50° in relation to horizontal plane). Setting of plates in the oblique position in the centre of the well bottom creates a liquid-air inter-phase while the formed biofilms (after plate well painting and rinsing) can be simply analysed by invert microscope (phase contrast microscopy). If the laboratory does not dispose with invert microscope, it is possible to place plastic or glass cover flakes in the plate well so that liquid-air inter-phase is achieved at their middle. After painting and rinsing with crystal violet the formed biofilms on the border of two physical states can be analysed using classical microscope.

11.3. Colony-based biofilm system

In this technique, a semipermeable membrane used for biofilm formation is set on the surface of the substrate (agar) for bacteria cultivation. Nutrients are provided by moving the membrane onto a fresh agar. In this system, the growth of bacteria is better since nutrients are continuously provided and there is no detachment of cells typical for biofilms immersed in liquid. Stable nature of biofilms formed in such a way is particularly useful in testing of their sensitivity to antimicrobial agents. In that case, the reduction of the number of bacterial cells can be more reliably attributed to the cell death rather than to detachment.

The advantages of microtiter plate biofilm assay include its simplicity, speed, and use of small quantities of chemicals, while the possibility of simultaneous work with a large number of isolates makes it ideal for the use in screening purposes. The implementation of this test enables simple varying of several parameters: composition of the medium, pH, incubation temperature, presence or absence of shear forces, O2 and CO2 concentrations. That is why this test has been frequently modified for different studies such as the impact of surface impregnation on different stage of biofilm development, antimicrobial effects of different substances and antibiotics, disinfectants and plant extracts [65]. The variations in factors (concentration of crystal violet, type of solvent, length of incubation) that were implemented in testing of biofilms within the same specie aggravate the comparisons of the obtained results. That is why the main disadvantage of microtiter plate biofilm assay with the application of crystal violet is the absence of the official protocol within which it is necessary to define conditions for testing of biofilm formation for each microorganism. In addition, crystal violet is the basic colour that binds onto negatively charged and surface molecules and polysaccharides of extracellular matrix as well as bacterial cells (dead and alive). Therefore, it can be said that this test determines the total biofilm mass [66]. When effects of antimicrobial substances on biofilm are tested, an identical test should be conducted in parallel, with the addition of redox indicators (resazurine or tetrazolium salt) in order to analyse the effect of the tested substances on metabolic activity of cells in biofilm [67]. Independently from the applied method, it is recommended to perform visualisation of the formed biofilm using some of the high resolution microscopy techniques such as scanning electronic microscopy (SEM), transmission electronic microscopy (TEM), or confocal laser microscopy (CLSM).

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