Toxicology

The phenomenon of biofilm – conditions of its formation and functioning in an environment

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Summary

Numerous branches of microbiology pay their attention to the structure and functioning of a biofilm. This concerns mainly these branches of microbiology which are used in food, cosmetic and pharmaceutical industries, in processes of water treatment, biodegradability to medical microbiology. In the natural environment, microorganisms rarely occur as single and scattered cells referred to as plankton, but they are rather found in the form of biofilm [1]. In natural flowing waters like rivers, the ratio of bacterial cells in a settled form to cells in the form of plankton is 1000:1. Bacterial biofilm is formed by cells of microorganisms belonging to one or even to dozen or so species which are capable to adhere to one another and/or to a solid surface of various biomaterials. A factor which promotes a stabilization of settlement is the microorganisms' ability to produce extracellular mucus, so called glycocalyx. Cells which form biofilm produce relatively thin matrix containing polysaccharides, nucleic acid and proteins [2]. In such a compact form surrounded by mucus, there are conditions in which cells of microorganisms are protected from unfavorable external factors, e.g. disinfectants, antibiotics, etc.

Both autotrophs and heterotrophs, including saprotrophs as well as infectious microorganisms, are capable to produce biofilm. Apart from bacteria, a biofilm can consist of fungi, protozoa or algae. The structure of biofilm can function in conditions in which single cells would hardly survive or would not survive at all. The reason for such a situation is that cells of microorganisms which are included in a biofilm are very able to perform various functions and they reveal different features compared to cells living in a water form. The structure of such clusters protects microorganisms from unfavorable influence of external factors and offers a possibility to access nutrients more easily.

The variety of phenomena connected with formation of biofilm contributes to the fact that learning its structure, mechanism of its formation and functioning becomes necessary in order to improve efficiency of technological processes which are conducted with the use of biofilm in the cosmetic industry. Another purpose of such a study, however, is to develop efficient methods to combat biofilm, curb its development and prevent this phenomenon to occur. It can be beneficial in regard to probiotics, but it causes numerous problems in industry and medicine.

Key words: biofilm, Quorum sensing, Gram-negative and Gram-positive bacteria.

Formation and development of a biofilm

Formation of biofilm is a multistage process dependent upon properties displayed by microorganisms which are included in it and the structure as well as properties of materials which are colonized, or, relatively of a colonized host. A living organism can become a host. Formation of a bacterial biofilm includes a few consecutive stages, e.g. adhesion, creating microcolony and then transformation of the settled bacteria into a mature biofilm. Formation of a biofilm begins with the attachment of a free-floating microorganism to a surface in an irreversible manner. What plays a significant role in the process of adhesion, are intracellular polymers, lipopolysaccharides and proteins of their cell wall, as well as extracellular structures, e.g. fimbriae and cilia [3]. The structure of the surface as well as its unevenness and damages make the colonization easier. The process of biofilm formation has not been fully explained yet. What is observed in the stages of biofilm formation, is a phase of reversible microorganisms adhesion, irreversible adhesion, maturation of biofilm and dispersion phase (Figure 1) [4].

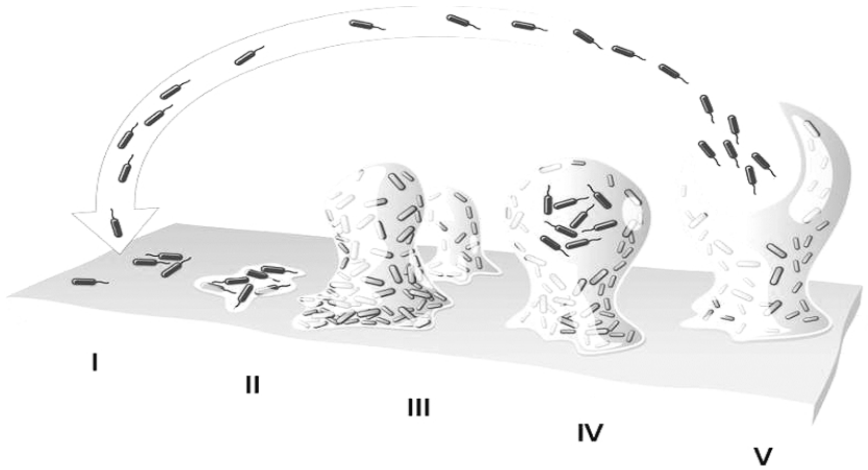

Figure 1: A diagram presenting stages of the formation of a bacterial biofilm.

STAGE 1: bacteria settle on the surface, beginning the process of gene expression which enables communication between them;

STAGE 2: bacteria adhere closely to the surface;

STAGE 3: units of bacteria come into being. They form microcolonies;

STAGE 4: maturation of the biofilm and transformation of bacterial units into complex forms of con or mushroom shape;

STAGE 5: single bacteria or entire units are delivered into surrounding environment.

A mature bacterial biofilm develops in a process consisting of five stages. Initially, the motion of cells in the direction of the surface supposed to become settled is regulated by physical effects connected with hydrodynamic, gravity and thermodynamic powers based on Brownian motion and van der Waals forces. The forces described above play a significant role in reversible adhesion, where the distance between cells and the structure supposed to become settled is relatively huge. In terms of this initial process of biofilm formation, the biofilm may be easily removed with the use of physical and chemical means.

Forming the first film in the structure of biofilm results in so called network thank to which free places in an abiotic surface are taken by consecutively settling cells. Bacteria are subject to transformation. Projections whose structure is similar to the one of hair come into being on the surface of bacteria. In regard to first stages of adhesion process, an active approachment of a bacterial cell to a solid surface with the use of bacterial instrument – cilia – is found significant. Cilia act as a motile force which is able to overcome the forces repealing between negatively charged surface of a cell and target surface. Strains with cilia attach to abiotic surfaces much faster and more efficiently than the ones without cilia. In the case of, e.g. *Pseudomonas aeruginosa*, this process is approximately 3 times faster [2,3]. Proper tracking microscope techniques allow to observe and measure the speed and track of the motion of bacterial cells in vicinity of inanimate flat surfaces to which the said cells can adhere in a non-peculiar manner. In regard to Escherichia coli, the observation of this phenomenon showed that these bacillus move with the constant speed at circular routes during one minute when they are near to glass surfaces. The speed of the motion of the bacteria decreases when the distance amounts to 10μm from the surface. It decreases the faster, the closer the cell is to the target surface.

The second stage of adhesion occurs when the distance between the cells and the abiotic surface is smaller than 1.5 millimetres. This is an irreversible process. Specific chemical bonds come into being between the surface which is being settled and adhesins being on the surface of the cells. The adhesins mentioned comprise flagella, pili, fimbriae and polysaccharide polymers. Specific chemical bonds contribute to formation of hydrogen bonds, complexes with covalent bonds of a carbon-carbon type. Extracellular polymers play a significant role in this process and they form so called glycocalyx. Thanks to this polymer, it is possible for microorganisms to adhere to abiotic surfaces as plastic or metal. Initially, the substrate covers with a single layer of cells of microorganisms. During this time, a synthesis is observed as well as an increased production of extracellular biopolymers, while the cluster of microorganisms surrounded with mucus stimulates adhesion of other microorganisms.

Irreversible adhesion allows production of microcolonies and maturation of biofilm. In this cycle, microorganisms multiply and diversify. During this process, the changes of settled populations of bacterial cells into highly organized structures of biofilm, the cells begin the process consisting in synthesis of new proteins. These proteins are different from the ones which are synthesized in similar conditions of growth by plankton cells. It has been determined that the use of tetracycline in the concentration which does not cause plankton cells to die in order to curb the proteins synthesis, curbs the process of *Pseudomonas fluorescscens* biofilm formation. Induction or suppression of synthesis concerning a few proteins has been observed in subsequent stages of *Pseudomonas aeruginosa* biofilm development on the glass wool surface [3].

A mature form of a biofilm includes a huge number of microcolonies which are separated from each other with a system of small canals thank to which nutrients are delivered to the upper parts of biofilm, while redundant metabolic products are excreted. In the case of bacteria which are immobilized in the biofilm matrix, the production of flagellin is stopped what results in disappearance of cilia. Bacteria living in the internal part of biofilm are exposed to the limitation of the access to oxygen what influences their metabolism. Activity of anaerobic metabolic pathways, such as desulfurication, denitrication and fermentation, increases while proteases synthesis and phospholipase C and toxins decreases. Thanks to the processes mentioned, cells which are included in the structure of a biofilm have different features compared to the cells living in a water form. Various genetic changes proceed which are transferred to next descendant cells living in the biofilm. Its mature form is surrounded by a thick layer of glycocalyx to which mineral substances, organic compounds and cells of other microorganisms are absorbed. The last stage of biofilm formation proceeds when its layer achieves its critical thickness what leads to decomposition of the existing structure. During this process, cells from preferential areas of a mature biofilm migrate to environment. It is supposed that separation of cells from a biofilm as well as its dispersal is intentional and it results from the reaction to unfavourable environmental changes. This may be caused by exhaustion of nutrients or a problem resulting from the flow of nutrients in the structures of biofilm. Probably, this is how biofilm adapts to environmental changes and how separated cells begins to colonize new surfaces.

Functioning and structure of a biofilm

A biofilm is a structure which can have one or more layers. In the second case, such a layer can consist of one or more species of microorganisms [5]. The entire architecture of a biofilm depends on numerous, various factors, i.e. contents of nutrients, hydrodynamic conditions, motility of bacteria, intercellular communication, contents of polysaccharides and proteins.

The table presents a function and content of exocellular polymeric substances (EPS) which perform numerous significant functions in the process of biofilm formation.

otic microorganism takes the ecological niche in the ecosystem of the stomach or intestines (otherwise, this place could be taken by a pathogen). This phenomenon is known as competitive exclusion and it was described by Stavric. According

Table 1: Fundamental functions in the structure of biofilm of exocellular polymeric substances (EPS) [5].

Element of EPS	Function of EPS
Proteins, polysaccharides, DNA	Adhesion of plankton cells to biotic and abiotic surfaces
Proteins, polysaccharides, DNA	Mutual recognition, increase in density and aggregation of bacterial cells
Proteins and polysaccharides	Maintaining mechanical stabilization of biofilm and intercellular communication
Proteins and hydrophilic polysaccharides	Maintaining hydrated environment within the structure of biofilm, ensuring tolerance to drying of cells
Proteins and hydrophilic polysaccharides	Sorbiton of organic compounds allowing accumulation of nutrients and sorbiton of xenobiotics
Polysaccharides and proteins containing phosphates and sulfates in their structure	Production of polysaccharide gel, accumulation of toxic metals
Proteins	Enzymatic activity allowing degradation of EPS to setting free cells from the biofilm. A factor which influences virulence of the biofilm in an infectious process
Probably all elements of EPS	Using various sources of carbon, nitrogen and phosphorus as nutrients' substrates by microorganisms forming the biofilm
DNA	Exchange of genetic material by transfer of genes between cells in the biofilm

Composition of EPS is different in Gram-negative bacteria compared to Gram-positive bacteria. Providing microorganisms which form the biofilm with nutrients and oxygen as well as excreting metabolic products is possible thanks to the network of small canals. Microcolonies separate the canals from each other.

Adhesion of probiotic bacteria

Adhesion abilities become one of the most desired feature of probiotic bacteria. Due to this fact, these bacteria can stay adhered to a human digestive track for a long time. Consequently, their beneficial influence on a human being is prolonged. The majority of bacteria which are included in the microflore of digestive tract develop in closed microcolonies attached to cells of epithelium or food particles in stomach and intestines. Without adhesion abilities and abilities to perform colonization, a particular probiotic strain will act favourably on the organism of the host as long as it is staying in the digestive tract. If, however, colonization proceeds, then a probi-

to him, introduction of intestines microflore of a healthy hen into chickens will protect them from infection with salmonella sp. A similar effect can be achieved by controlling infections triggered off by other pathogens, e.g. *E. coli*, *C. difficile*. In the case of piglets who were fed with feedstuff containing *Lactobacillus lactis* a smaller number of pathogenic *E. coli* strains was found compared to the group of animals which did not take the probiotic. It is also known that Lactobacillus acidophilus is subject to adhesion to Caco 2 human cellular line. Such a situation may, in.al. prevent enteropathogenic bonding of the *E. coli* and Salmonella typhimurium strains with cells coming from the culture and does not allow *E. coli*, *Y pseudotuberculosis*, *S typhimurium* to get into the cells of the host.

The data described show that it is possible to determine microflore of digestive tract which would be favourable to the host as well as to prevent pathogenic bacteria from settling into mucous membrane of intestines due to the use of probiotics. A favourable influence of probiotics on health of humans constitutes a significant element in research on colonization of intestines' mucous membrane by microorganisms. Probably, it is connected with oligosaccharide of intestines' walls bonding with bacterial lectin. The mechanism is presented in Figure 2. [in an original work 1]

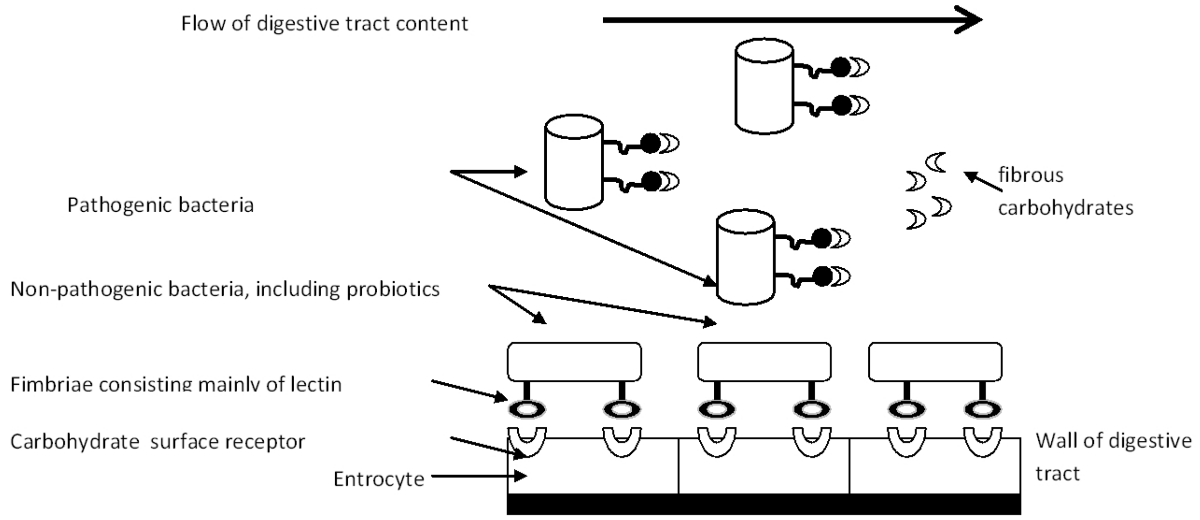

Figure 2: A scheme presenting a possible mechanism for colonization of mucous membrane of intestines by probiotic bacteria. A possibility to stop pathogenic bacteria to colonize the walls of intestines by combining polysaccharides delivered with nutrients and lectins being on the surface of pathogenic bacteria.

Combining polysaccharides delivered with nutrients and lectins being on the surface of pathogenic bacteria leads to blocking the possibility of colonization by the pathogen in digestive tract, while non-pathogenic bacteria, e.g. probiotics can adhere to this place in mucous membrane of intestines.

Quorum sensing phenomenon vs. formation and functioning of biofilm

This phenomenon constitutes a huge surprise for researchers who have been convinced for many years that such microorganisms like bacteria do not have any systems of their own communication. Currently, it is known that this phenomenon concerns chemical communication of microorganisms and consists in production and liberating signal particles to the environment which are used in various physiological processes, in.al. formation of biofilm [6]. First data regarding possibilities of bacteria's communication date back to 1979 when the phenomenon of bioluminescence of marine bacteria was described. It concerned bacteria which are able to emit light only when they live in organisms of marine animals. They lose this ability when cells stay in water as freely living creatures. The research conducted indicated that microorganisms produce chemical signals referred to as autoinducers whose increase in concentration depends on the number of developing bacteria population. It seems that chemical communication of microorganisms may be of great importance in regard to formation of infection, formation of biofilms from microorganisms with high tolerance to antibiotics.

Currently, approximately 30 species of bacteria in regard to which *the quorum sensing* phenomenon occurs are known. The ones which are known to the biggest extent are *Vibrio fischeri*, *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, *Erwinia cartovora*. The manner of communication between microorganisms is different dependent upon the structure and chemical structure of the cellar wall of Gram-positive and Gram-negative bacteria [7].

The scientific research showed that microorganisms send communication signal not only within one species, but between various species as well.

The molecular mechanism quorum sensing allows the cell of bacteria to produce signal compounds and to accumulate them in an environment. Consequently, it allows the cells of bacteria to recognize the signals by specific receptor proteins which leads to expression of gens which control significant life processes. Bacteria can recognize the chemical character of signals with high precision and their threshold concentration in the environment of growth which allow to control physiological-metabolic process of entire population. Dissimilarity of structure of Gram-positive and Gram-negative bacteria constitutes a difference in the system of transferring signal particles. In regard to Gram-negative bacteria, functions of autoinducers are performed by acyl homoserine lactones (AHL), while in regard to Gram-positive bacteria, this role is taken by specific oligopeptides. There are also autoinducers which are non-specific for species and which allow to communicate at the molecular level of microorganisms belonging to species that are often philogenetically distant to each other.

Communication of Gram-positive bacteria

In order to communicate, Gramm-positive bacteria use proteins particles with twofold detection system and system for response to the presence of an autoinducer. This process is presented in Figure 3.Autoinducing polypeptides (AIPs) come into being as a result of digestion of bigger protein precursors. In contrast to AHL of Gram-negative bacteria, the communication particles of Gram-positive bacteria are not able to diffuse by cellular shields. The particles are produced outside thanks to transporting proteins dependent upon ATP. At the moment when they achieve the right level of concentration, they adhere to protein kinase connected to cell membrane. In the phosphorization process, an autoinducer is recognized. In the response, the protein regulator may bond the DNA promoter and regulate the expression of suitable genes. Dependently on the species, the following can be autoinducers for Gram-positive bacteria: oligopeptides, cyclic octreotides, sidephores or butyrolactones.

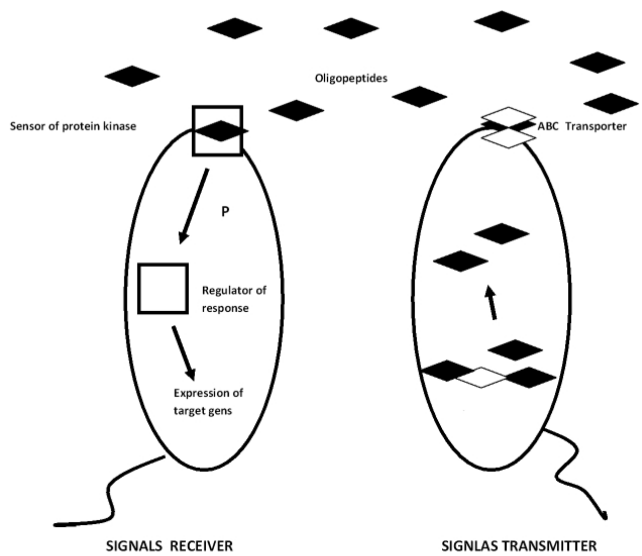

Figure 3: The scheme of intercellar communication of Gram-positive bacteria. Elaborated on the basis of [8,9].

Communication of Gramm-negative bacteria

The Quorum sensing phenomenon was discovered among more than 25 species of Gramm-negative bacteria. In the case of the bacteria mentioned, the communication manner is expressed by N-N-acylohomoserine lectine particles autoinducers whose synthesis depends on the protein similar to Lux1. The ring of homoserine's lectine is acyled in the α position of fatty chain. Individual signal particles are different in terms of structure of fatty acid and atom number in its chain. Such a situation allows each species of bacteria to have its own "language" for communication which cannot be understood by microorganisms belonging to other species. Chemical dependencies of this process are presented in Figure 4. Autoinducers are provided with moieties which can freely diffuse through cellular shields, while the ones which contain longer moieties (more than 6 carbon atoms) for transportation into internal environment use proton pump. Acyl group originating from processes of fatty acids and S-Adenosyl methionine (SAM) is used to produce AGL particles. Acyl carrier protein functions as a transporter of moiety on a lectin particle. The synthesis is led by proteins from the family of synthase of LuxI autoinducer. S-Adenosyl methionine binds with the active centre of an enzyme and, subsequently, the acyl group becomes bound with SAM acyl bond. As a result of this reaction, ester bond comes into being in homoserine and methylthioadenosine is produced which allow acyl homoserine lactones (AHL) to come into existence. AHL particles can defuse freely in the structure of biofilm from one cell to another but only when a proper concentration in an environment is achieved, it is possible to interpret the chemical signal. LuxR protein transcriptional regulators function as detectors. Binding an autoinducer's particle with LuxR protein is the reason for changes in conformation, activation and induction of target gens. Thanks to these processes, the expression of the proper gens allows cells which are included in the biofilm to function in new conditions.

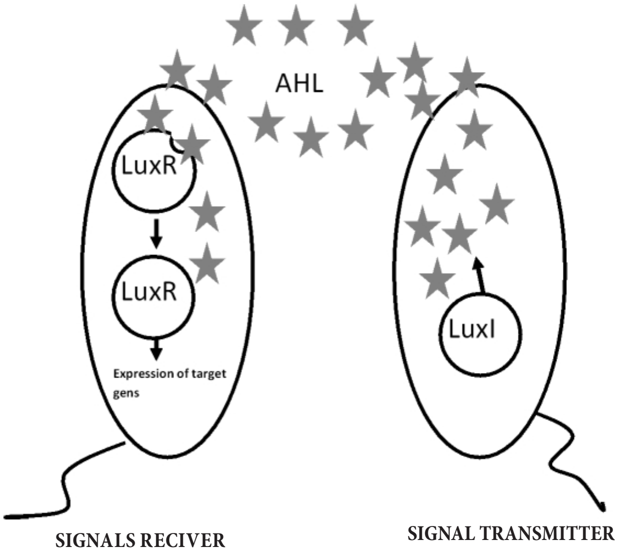

Figure 4: Scheme of intercellular communication of Gramm-negative bacteria. Elaborated on the basis of [8,9].

Biofilm resistance to the substances of antimicrobial nature

Cells of microorganisms accumulated in a biofilm are more resistant to effects of external conditions compared to the ones which live in a water form. [10]. It is caused in.al. by EPS polymer, which – thanks to its viscous structure – surrounds the biofilm cells and constitutes a protective barrier. Due to its properties, EPS limits diffusion of microbial substances into the internal parts of biofilm and decreases efficiency of immune cells functioning in the body of the attacked host. Furthermore, EPS protects microorganisms from phagocytosis and drying.

As biofilm grows and becomes mature, there are more and more polysaccharide substances in its shield what increases the number of free functional groups and contributes to high resistance of the said microorganisms. When a biofilm is influenced by antimicrobial substances, the role of EPS in the resistance phenomenon due to lack of free functional groups in egzopolysacchaide bacterial chains. [5,11,12]. The influence of antibacterial substances may be also affected by conditions existing in the internal biofilm structure. The decreasing access to oxygen what regards deeper layers of biofilm leads to the situation when cells from deeper biofilm parts go into anabiosis. Hence, their multiplying within the internal parts of biofilm is slower.

Bacteria in a biofilm became specialized in producing special proteins which protect them from effects of external factors. As a result of high temperature, toxins, radiation, starvation, they experience expression of gens which are responsible for induction of production of special proteins, i.e. heat shock proteins. They play a significant role in regard to protecting vital protein cells. They surround the cells mentioned and protect them from irreversible changes which could result in the cell's death.

A long-term influence of antibacterial substances on a biofilm may also cause induction of point mutation of genes whose expression products increase the resistance level of individual cells existing in the biofilm. In this structure it may come to horizontal transfer of genes. Transferring plasmids is a significant mechanism of spreading resistance to medicines, disinfectants or to other chemical substances [1].

Currently, there are four hypothesis which explain resistance of cells growing in a biofilm environment to antibacterial substances:

1) muscus egzopolysacchaides modify or block the access of antibacterial substances to the cells of microorganisms;

2) bacterial cells on the surface of the biofilm or muscus egzopolysacchaides react to other active groups of antimicrobial substances, decreasing their efficiency and access to deeper lying layers of bacterial cells;

3) limiting availability of basic metabolism substrates to the deeper lying layers of cells stimulates such changes to arise in phenotypic cells which live there to such an extent that

their sensitivity to antimicrobial substances is considerably decreased

4) adhesion of bacteria to solid surfaces causes induction or expression of genes which have not been known yet and which are different than the genes active in twin freely-living cellar genes being subject to suppression in plankton cells.

Biofilm – problems resulting from settling various types of surfaces

Scientific research has paid much attention to phenomena connected with adhesion of microorganisms to various types of surfaces recently. This fact should be deemed as unfavourable in much extent: microbial corrosion, contamination of surfaces having contact with food or cosmetics produced, hospital infections [13,14]. It is also possible to discuss the positive aspect of adhesion ability of bacteria – colonization of intestines by probiotic bacteria, fermenting processes used in technology and food and feedstuff production, share of microorganisms in production of humus in soil.

Microorganisms which produce a biofilm constitute a serious problem with which the cosmetic industry contends. They may settle both in cosmetics and in operating areas which are used to produce them. Infected products of plant or animal origin may be a reason for serious human illnesses. Microorganisms multiply on inanimate surfaces and then on skin or in digestive tract in the form of microcolonies surrounded by a layer of glycocalyx which is difficult to combat with chemical compounds.

In cosmetic industry, covering of the operating area by a biofilm may lead to the infection of cosmetics with microorganisms which can cause cosmetics to go bad, as well as infection with infectious microorganisms. Bacterial biofilms colonize efficiently surfaces made of stainless steel, glass or teflon. The research conducted shows that the type of the microorganism studied and availability of nutrients correlates with the speed of biofilm formation [15,16]. These phenomena are the reason for serious losses for cosmetic industry.

Another problem constitute greases and oils used in regard to numerous devices and engines, not only the ones used in the cosmetics production [16]. Taking into account the hydrophobic nature of oils and greases, they are not much prone to biodegradation. When the substances mentioned come into contact with water, air or microorganisms, the situation becomes problematic. On the border of oil-water phase, microorganisms develop and a thin layer of biofilm comes into being. A part of microorganisms elaborated mechanisms thanks to which they are able to use hydrocarbons as a substitute of nutrients. Biodegradation of hydrocarbons is carried out by numerous species of bacteria and fungi. Also microorganisms which are infectious for humans belong to this group. A result of microorganisms degradating the hydrocarbons are problems with functioning of numerous devices which is caused by changes in physical and chemical properties of greases. Microorganisms biodegrading the hydrocarbons may cause the production of organic acids as products of decomposition which cause not only metal corrosion, but they also have also negative influence on structure, consistency and quality of the cosmetics produced [15,16].

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