ABSTRACT

The development of industry causes serious changes in the composition of water, soil and air, which in turn leads to the breach in the ecological balance. This problem is topical for the whole world, determining the need to introduce cleaner technologies and more effective treatment of waste. It leads to a policy on the control status of components and environmental factors.

Essential for the normal functioning of all ecosystems is the question of wastewater treatment. Bioremediation is an innovative technology that controls the contamination. It uses biological systems, which catalyze the degradation or transformation of various toxic chemicals. In recent years a major role in bioremediation is of biofilms. They are used as the cleaning of wastewater from toxic contaminants and heavy metals, and in the process of methanisation. Some biofilms are useful, providing valuable services to human society and functioning of natural ecosystems. Other biofilms are harmful, causing serious health and economic problems. The study of the mechanisms of the biofilm formation, growth, or their removal is the key for producing useful and reducing harmful biofilms.

Keywords: biofilms, bioremediation, pollutants, wastes.

INTRODUCTION

The majority of microorganisms in nature, industrial and clinical environments are associated to a surface state, interact with each other and form a seamless integrated environment called biofilm. By definition, biofilm is structurally and functionally distinct communities of microorganisms organized on a variety of natural and artificial surfaces [1]. Growing importance in the economy, health and ecology on the one hand and the complex structure on the other led to intense interdisciplinary research on biofilms with participation of specialists from different fields: biotechnologists, microbiologists, chemists, engineers, physicists and others.

Biofilms are used in industry and ecology. Biofilms are effectively applied in wastewater treatment, industrial water and air pollution, and others.

Although most pollutants from the chemical nature have an inhibitory effect on the growth of microorganisms is proved that biofilms formed by different strains are able to overcome this effect and to degrade these contaminants using them as carbon sources [2].

FORMATION OF BIOFILMS

The formation of bacterial biofilms must, necessarily, begin with the adhesion of a small number of bacterial cells to a surface. The main difficulty emanates from the tendency of the microbiologists to use laboratory-adapted bacterial strains, whose behavior they can anticipate, in contrast with “wild” strains fresh from their natural habitats. Many laboratory-adapted strains have virtually lost their ability to adhere to surfaces because, each time they are transferred into fresh medium. The inoculum for this operation is taken from
the bulk fluid and any bacterial cells that are adherent to the walls of the vessel are left behind. After a few hundreds of transfers of this kind, most laboratory-adapted bacterial strains have been heavily selected for rapid growth and against adhesion to surfaces [3].

Wild bacterial strains must survive in nature, among a host of antibacterial factors, and they need their protective outer layers even more urgently than they need to adhere to surfaces. Wild bacterial strains, in real ecosystems, have a thick layer of extracellular polymeric substances (EPS) surrounding each cell, through which long fimbriae protrude to form an effective part of the cell surface [3].

Revelations of the basic architecture of biofilms have shown that the microcolony is actually the basic structural unit of the biofilm. The exhaustive structural analysis of hundreds of monospecies in vitro biofilms, and of dozens of multispecies natural biofilms, has shown that microcolonies are discrete matrix-enclosed communities of bacterial cells that may include cells of one or of many species. Depending on the species involved, the microcolony may be composed of 10–25 % cells and 75–90 % EPS matrix, and the matrix material often appears to be most dense in the area closest to the core of the microcolony. Bacterial cells within the matrix are characterized by their lack of Brownian movement, and careful structural analysis of the shape of many microcolonies often reveals a mushroom-like shape. Most of the cells are in the “crown” of the mushroom and very few are in the “stalk”. Microcolonies are, of course, arranged in a horizontal array in thin biofilms, but they may also form vertical arrays in very thick sessile communities [3].

The structure and formation of biofilms is the subject of research and of great interest to many scientists. It is known many media that can be used as substrates for biofilm development, as polysaccharide matrices and synthetic copolymers based on styrene, maleic anhydride, divinylbenzene, and block polymers of valerolakton [4].

The formation of biofilm has several stages (Fig. 1.).

The first stage is when the microorganisms are in contact with surfaces and develop a microcolony thus forming the “safety layer” (conditioning biofilm). The next step is the adhesion of microorganisms to this layer. Bacterial adhesion is a reversible process and is implemented using flagella and EPS, which serve as a bridge between bacteria. Initial links between bacterial cells and EPS are not very strong and they can easily fall into the flowing stream. Over time, these connections are strengthened and the attachment becomes irreversible. The third phase consists in the increase of the biomass. During the fourth and the fifth phase, the bacteria grow and multiply, secreting large amounts of EPS providing a protective barrier of cells. In addition, in the fifth stage, there are prerequisites for the separation of microorganisms from the biofilm system and transfer them to another area where new biofilms are formed [5].

**SPREADING OF BIOFILMS IN THE NATURE**

Biofilms exist everywhere. Almost all organisms, not only bacteria and archaea bacteria have mechanisms that can attach to surfaces and each other.
Biofilms can be found on rocks, pebbles, and often on the surface of stagnant pools. In fact, biofilms are an important component of the food chain in the rivers and the streams consumed by some river invertebrates, which in turn are a food for many fishes [6].

Biofilms may be a useful, constructive purpose. For example, many businesses sewage purification include a phase in which contaminated water passes over the biofilms placed on the filters and so nutrients are extracted and used by the microorganisms of the films. In such water treatment bacteria are mainly responsible for removal of organic matter, while Protozoa and Rotatoria are responsible for the removal of suspended salts also pathogenic and other microorganisms. Sand filters rely on biofilm development for the same reason, the purification of water from lakes, springs or the sources for drinking water [7].

EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

Biofilms are a collection of microorganisms surrounded by the slime they secrete, attached to either an inert or living surface. You are already familiar with some biofilms: the plaque on your teeth, the slippery slime on the river stones, and the gel-like film on the inside of a vase that held flowers for a week. Biofilm exists wherever surfaces contact with water [8].

Biofilms retains its integrity and is protected by a matrix composed of microorganisms isolated from polymeric compounds called extracellular polymeric substances (EPS). Other compounds in the composition of the matrix are extracellular polysaccharides, proteins, uronic acids, nucleic acids, lipids and others. This matrix protects biofilm cells, enhances and facilitates relationships between them through biochemical signals. There are open water channels in some biofilms that help the distribution of nutrients among the biofilm cells. Extracellular polymeric substances are high molecular mass polymers with a molecular mass between 500 and 2000 kDa. They can be associated with one another, interact with other components of the matrix itself, as proteins, lipids, ions and other macromolecules from the bacterial cell surface. So they can form a polymer network, which determines viscosity of the biofilm state. The extracellular matrix varies widely in composition, structure and properties and it is impossible to summarize its contribution to the formation and properties of the biofilms [8].

SURFACE/MATERIAL

A major factor influencing the biofilm development in water treatment systems is the surface area. Industrial water systems, unlike most natural environments (lakes and rivers), offer a tremendous amount of surface area for attachment. Different polymeric membranes, resins, storage tanks, cartridge filters, and piping systems provide surfaces suitable for bacterial attachment and growth [9].

There are known several microbial strains which can form biofilms onto various matrices: Bacillus cereus, Arthrobacter species, Bacillus licheniformis, Halomonas salina, Bacillus pumilus, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas species 1625, Candida albicans, etc. [10-14].

From the above strains in our studies were selected three types of microorganisms involved in the formation of biofilms on newly synthesized hybrid membranes as follows: Arthrobacter oxydans 1388, Pseudomonas species 1625 and cells of bakery yeasts-Saccharomyces cerevisiae. It is known a covalent method, successfully applied for immobilization of Gram-positive microorganisms such as Arthrobacter simplex [15] and Arthrobacter oxydans [16] to cellulose granules. This method consists of sequential steps of activation, including treatment of cellulose with periodic acid, urea and finally with formaldehyde, thus creating hydroxymethyl groups able to link with amino groups of the cell wall of bacteria.

It is known binding of cells from Arthrobacter oxydans 1388 by adhesion to the surface of 4 types of synthetic membranes based on block copolymer valerolakton [17], polymethylacrylate (PMMA), copolymer of acrylonitrile acrylamide (AN-co-AA) and copolymer of acrylonitrile acrylamide and cellulose acetate butyrate (CAB), ((AN-co-AA)+CAB). The method of immobilization by adhesion is relatively simple and easy to perform, applicable to more sensitive cells. During the research it was found that this method of attachment of microorganisms and the formation of stable biofilm depend on various factors: the nature of the cell surface, the age of the cell culture, the ionized groups, the type of polymer matrix, etc. These parameters significantly affect the adhesion of cells. Changes in these factors might lead to a desorption of microorganisms.

This study is also directed to follow the formation of biofilms of cells of Gram-negative bacteria-
nas species 1625 and yeasts cells-Saccharomyces cervisiae. As carriers for the formation of biofilms as carriers are composed selected matrices (AN-co-AA)+CAB and co-polymer of acrylonitrile (AN), acrylamide (AA), cellulose acetate butyrate (CAB) with an inorganic network by incorporation of titanium alkoxide, ((AN-co-AA)+CAB+TBOT), on the grounds of the positive experimental results in the development of biofilms of cells of Arthrobacter oxydans 1388 on matrix based of the (AN-co-AA)+CAB. The results of biochemical studies of biofilms formed by Gram-negative bacteria and yeasts on both matrices show that the accumulation of proteins and polysaccharides is proportional to the incubation period of the development of biofilms in each of the matrices used separately. The growth of biofilms on both matrices is also determined by the time of development. At the same time, the comparison between the rates in both matrices showed a steady increase in the matrix consisting in metal precursor.

APPLICATION OF THE OBTAINED BIOFILMS

Phenol is toxic in very low concentrations. It is therefore necessary to apply different methods to reduce the phenol concentration in wastewater to normative values. Biological treatment of water is the most attractive. Phenol degrades to harmless end products and secondary mineral wastes [18-21].

Biodegradation of phenol was studied for a long time and several studies show that the aerobic degradation of phenol is carried out using a wide variety of microorganisms including bacteria such as: Arthrobacter chlorophenolicus [22], Pseudomonas pictorum [23], Pseudomonas aeruginosa and Pseudomonas fluorescens [24,25], Acinetobacter Sp. [26], Nocardioides sp. [27], and some yeasts such as Candida tropicalis [28,29] and Trichosporon cutaneum [30-32].

We have studied the application of biofilm cells of Pseudomonas species 1625 formed on two types of matrices (AN-co-AA)+CAB and (AN-co-AA)+CAB+TBOT in the processes of biodegradation of phenol and benzyl alcohol, present in the nutrient medium and in the mixture of the toxic compounds.

From the literature it is known the application of biofilms of two xenobiotic-tolerant bacterial isolates-KCM R5 and KCM RG5, formed on the cryogens in the process of biodegradation of phenol. Researchers examined the ability of biofilms formed to degrade phenol at concentrations: 300, 400, 600 and 1000 mg l⁻¹ within 28 days. Biofilms formed by cells of KCM RG5 remove 600 mg l⁻¹ phenol within 24 hours, while biofilms of KCM R5, biodegrade for 24 hours the highest concentration of phenol-1000 mg l⁻¹ [33].

We studied the ability of Pseudomonas species 1625 biofilms to remove phenol in three different concentrations 0.1 mg ml⁻¹, 0.3 mg ml⁻¹ and 1 mg ml⁻¹ respectively within 3 days of incubation. Comparison was made between the free cells and the biofilms formed on two types of matrices. During the experiments was determined that biofilms biodegrade minimum concentration of phenol of 0.3 mg l⁻¹, in the first 6 hours of incubation. Similar behavior demonstrate free cells, but degradation of phenol in the first 6 hours is less than that of biofilms. In considering the influence of phenol concentration in a 1 mg ml⁻¹ was found a toxic compound to be an inhibitor for cells growth both for free cells and biofilms. In both cases biodegradation was not observed.

The biodegradation of benzyl alcohol with concentrations 0.1 mg ml⁻¹, 0.3 mg ml⁻¹ and 1 mg ml⁻¹ by free cells and biofilms of Pseudomonas species 1625 formed on two types of matrices (AN-co-AA) + CAB and (AN-co-AA)+CAB+TBOT was also studied.

The experiment results show that the free cells degrade completely benzyl alcohol in all the concentrations after 36 hour of incubation. In biofilms, benzyl alcohol at higher concentrations (0.3 mg ml⁻¹ and 1 mg ml⁻¹) is completely degraded after 48 hour of incubation. In concentration of 0.1 mg ml⁻¹ it was observed to hold 50 hours. After that the benzyl alcohol was fully sorbed.

A comparison was made between the free cells and the biofilms of Pseudomonas species 1625 cultured on medium containing a mixture of phenol and benzyl alcohol in concentrations of 0.05 mg.ml⁻¹ for each of the toxic components. The results showed that the free cells of Pseudomonas species 1625 developed relatively well in the substrate medium. Similar behavior show the biofilms cultivated in the same environment. Benzyl alcohol is completely removed after 36 hours of incubation in both biofilms.

RHEOLOGICAL PROPERTIES OF OBTAINED BIOFILMS

There is a growing interest in the mechanical properties of biofilms that exhibit rheological behavior of fluids. Understanding these properties provide the
necessary basis for effective control of biofilms in industrial and medical environments. Researchers presented detailed viscoelastic properties of biofilms of cells of *Pseudomonas aeruginosa* [34]. Other researchers demonstrated that bacterial attachment depends on the critical surface tension of the solid surface [35].

In the present study are shown the measurements with quartz crystal microbalance (QCM) (Fig. 3). It is followed modules of accumulation ($G'$) and loss ($G''$), to the viscoelastic properties of newly synthesized matrix and biofilms formed by cells of *Pseudomonas species* 1625 and *Saccharomyces cerevisiae*.

Researchers follow the accumulation and losses modules to at elasticity and viscosity of yogurt. They found that the viscosity of the module shows higher values than the modulus of elasticity [36]. Similar is the case of the study, in which were examined the early stages of gelation of newly synthesized matrix based on the (AN-co-AA)+CAB and (AN-co-AA)+CAB+TBOT. From the experimental data found in the matrix containing only organic polymers in the composition have been observed higher values of the modulus of viscosity. Furthermore, when comparing the effects of different concentrations of Ti(OBu)$_4$ the unit of viscosity increased with the concentration of Ti(OBu)$_4$ [37].

According to researchers, the presence of Ti in the mixture significantly reduces the modulus of elasticity than the modulus of viscosity [38]. They follow modules of elasticity and viscosity area of the two types of matrices. One is based on silicon and the other contains Ti in its composition [38].

This study has looked at change modules and modules accumulation of losses based on the principles of viscoelastic theory, respectively modules of elasticity and viscosity. In the experiments is discussed variation of modulus of elasticity and viscosity, seeing as biofilms

Fig. 3. Set up of quartz crystal microbalance (QCM).
non newtonian fluids expressed initial values of elasticity [Fig. 4 and Fig. 5]. Similar studies are also known from the literature.

Researchers report about the module accumulation and module losses during the initial stages of biofilm formation of pathogenic cells of Streptococcus mutans as dental plaque. They found that relative to the characteristics of biofilms exhibits properties of organic polymers [39].

CONCLUSIONS

Synthesis was performed for selection of the most suitable hybrid membrane as a carrier for the formation of biofilms of three species of microorganisms.

Biofilms are formed by cells of Gram-positive bacteria-Arthrobacter oxydans 1388, Gram-negative bacteria-Pseudomonas species 1625 and yeast-Saccharomyces cerevisiae and were characterized the dynamics of growth and biofilm production of extracellular polymeric substances. It was traced the use of biofilms obtained in the process of degradation of toxic substances. Here was successfully investigated the rheological characteristics of biofilms using QCM.

Modern knowledge of the structure and the characteristics of biofilms significantly alter the approach of researchers in microbial ecology and biotechnology. This enables efficient use of biofilms in wastewater treatment.

Acknowledgements

The present work is funded and supported by project No213, FUND “Scientific Investigations”, Ministry of Education and Science, Bulgaria

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