THE ROLE OF CELLULOSE IN THE CONSERVATION OF HDPE BINDING SPECIFICITY TO POTENTIALLY PATHOGENIC BIOFILMS FROM THE AQUATIC SYSTEMS

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Abstract

In the last 20 years, moving bed biofilm reactor (MBBR) has been established as a simple-yet-robust, flexible and compact technology for wastewater treatment. It is one of the advanced aerobic wastewater treatment process by taking advantages of both attached and suspended growth systems. The vast majority of bacteria adhere to surfaces and form complex and heterogeneous microbial communities termed biofilms which have a tremendous positive potential in biotechnology for biocatalysis and waste treatment. In this work, we studied the Echerichia coli (E. coli) adhesion properties on synthetic artificial materials (SAMs), also known as biofilm carriers, based on High Density Polyethylene (HDPE). The results showed that E. coli adherence to SAMs is not only preserved, but it is also enhanced by the presence of 7% cellulose in SAM's composition.

Keywords: Adhesion; Bacteria; Biofilm; Moving Bed Biofilm Reactor

Introduction

Environmental pollution acts on many levels from decreasing the biodiversity at the local contaminated site to a climate change global level impact. To tackle this problem, more stringent pollution management and wastewater discharge standards have been adopted in recent years to protect the environment. Everyday more than 2-billion tons of wastewater originated from different sources are discharged into the natural water bodies. High-strength wastewater is a significant proportion of these wastewater and it is known to have an elevated concentration of organic substances, nutrients, suspended solids and other contaminants. Wastewater treatment importance is given by the need of increasing the effluents quality prior their discharge into environment, in order to reduce the pollution impact on the receiving bodies. Conventional wastewater treatment plants rely on physico-chemical and biological processes for organics and nutrients removal [1]. In a more specific way, the biological wastewater treatment step is majority carried on by microbial populations from the activated

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sludge composition. The structure of the microbial population is very dynamic, based on the pollutants chemical compounds released in the wastewater [2, 3]. The conventional activated sludge (CAS) – based process is not economical because the aeration energy requirement increases proportionally to the wastewater strength. A step forward from the standard wastewater treatment procedure is the usage of biofilms attached on various matrices which will enhance the treatment yield. Biofilms are spatially structured communities of microbes, which exchange chemicals and communicate with each other [4].

The bacterial populations from the biofilm's composition could be the leading cause of chronic infections [5], but at the same time they also have a tremendous positive potential in environmental monitoring [6, 7], biotechnology for biocatalysis, biofueling and waste treatment [8]. The use of biofilms in wastewater treatment systems starts with an initial adhesion of bacteria to matrices followed by a robust biofilms formation generating an effective removal of contaminants [9].

Biofilm development and formation is a very complex process. Biofilms can form on almost all types of materials in moist environments. Several stages were observed in the formation of the biofilm. Wet surfaces generally encounter a film of liquid containing adsorbed dissolved inorganic substances and organic molecules. The existing bacteria move to the surface with which the liquid film comes into contact, through Brownian motion processes, resulting in a temporary association between bacteria and the surface. Adhesion is a physic-chemical process mainly depending on attractive and repulsive forces, surface free energies, Gibbs free energy of adhesion and hydrophobic interactions.

Therefore, inter-bacterial adhesions, forming biofilms, involve physico-chemical forces, such as hydrophobic/hydrophilic interactions and interactions between strongly localized functional groups on bacterial cells and material surfaces; after the adhesion, the bacteria produce extracellular polymeric substances (EPS) to form the mature biofilm [8]. The process of surface attachment of bacteria is irreversible under static conditions and in the absence of other chemical inhibitors. Synthesis of exopolysaccharides represents a mechanism that leads to the irreversible attachment of the biofilm to the surface with which it comes into contact.

Biofilm formation on surface area relies on natural processes of cell attachment, EPS production leading of mature biofilm development and biofilm grow dynamics. Hence, microorganisms are structurally entrapped into cellular products present in a immobilizing matrix formed by EPS. They spontaneously develop into dense aggregates capable in adhering to a surface such as the one provided by biocarriers [10].

EPS compounds belong to the groups of macromolecules as polysaccharides, proteins, nucleic acids, glycoproteins, and phospholipids. Besides the inter-bacterial interactions, biofilms could interact with various surfaces and the literature mentioned that cell contact with surfaces stimulates transcription of the EPS genes. The biosynthesis of EPS has many functions linked to their promotion of the initial attachment of cells to solid surfaces, i) formation and maintenance of colony and mature biofilm structure, ii) enhanced biofilm resistance to environmental stress and disinfectants. In some cases, EPS matrix also enables the bacteria to capture nutrients. The EPS matrix is generally from 0.2 to 1.0μm thick. In some bacteria species the thickness of the EPS layer does not exceed values from 10 to 30nm [11].

During the initial contact between biofim and the surface, as mentioned before, different several forces are involved but also occur hydrophobic interactions. The first adhered cells seldom come in direct contact with the surface to which they attach due to the existing electrostatic repulsive forces, but these cells secrete polymers that are able to bind the cells to the surface.

The time from the reversible to the irreversible attachment is short, a few minutes. As soon as it attaches to the surface, cell division and the recruitment of planktonic bacteria begin. In this way the development of the biofilm on the surface takes place. Bacterial cells attached to the surface use nutrients in the environment to grow/develop and produce more extracellular

polymeric substances. By multiplying the attached bacteria, microcolonies are formed that develop continuously (in favorable environmental conditions) and finally form a continuous film, a film that will cover the contact surface [10].

Mature biofilms are heterogeneous structures, dynamic in space and time, which can take different architectural forms depending on the characteristics of the existing environment (pH, quantity and type of nutrients, temperature, other chemicals, liquid flow conditions, etc.). The composition of microbial consortia is also different [8]. Complex architectural structures, with the existence of "towers" of bacteria surrounded by water channels are very common. The water channels between the bacterial towers favor the transport of oxygen and nutrients to the bacteria situated at the base of the towers, of those cells located in the immediate vicinity of the contact surface. The process of developing a mature and robust biofilm is quite slow, often taking several days. In the WWTPs, in the absence of the addition of additional microorganisms, the mature biofilms on the biofilm carriers can form in periods of up to 4 weeks. A mature biofilm is a robust structure that continuously adapts to the environment, and in unfavorable conditions, bacteria can detach from the created biofilm in search of a new, more favorable habitat to attach, form and develop other mature biofilms. This step is known as detachment. In time and under certain conditions, both inactive and active cells detach from the formed biofilm. In wastewater treatment, the detached living cells form activated sludge, which can be recirculated.

One of the most used applications of biofilms is moving bed biofilm reactor (MBBR), which are operated similarly to the activated sludge process. MBBR is one of the methods of wastewater treatment, developed in the late '80s - early' 90s at the University of Science and Technology Norway, by *Hallvard Ødegaard* [12]. Compared to the conventional activated sludge process, this technology has the following special features: i) the functional bacteria grow on the protected surface of carriers, hence eliminating sludge recirculation, minimizing the solids in the effluent on the bioreactor and the size of secondary sedimentation tanks; and ii) the carriers are capable of freely moving in the reactor [13]. The performance and capacity of volumetric treatment in the clasical activated sludge treatment plant is improved in MBBR with minimal additional costs, the sludge does not need recirculation, because the biomass is kept as a biofilm on carriers and the biofilm is more resistant to variation of parameters (eg. shock loads, pH, temperature and toxic compounds). MBBR incorporates the advantages of both suspended and attach growth process were microorganisms grow on carriers as biofilm. The movement of the support material within the biofilm reactor is achieved either by the agitation produced by aeration or by mechanical stirrers. Biofilm grows attached on small carrier elements suspended in constant motion throughout the entire volume of the MBBRs [2]. The hydrodynamics from aeration (in aerobic tanks where the suspension of the biofilm carriers is ensured by the continuous elevation of the air bubbles) or mixing (in anoxic tanks where the carriers are kept in suspension by mechanical agitation), combined with the collision of the carriers in the system, ensures a good control of the biofilm thickness.

The biofilm established on carriers includes the existence of anoxic/ anaerobic inner layers and anaerobic outer layers. Therefore, the nutrient removal can be accomplished in a single reaction that reduces the land area requirement for wastewater treatment plant. The MBBR systems enable the upgradation of the existing wastewater treatment plants to increase the pollutants removal efficiency and reduce energy consumption. Numerous studies have shown that the MBBR system is an efficient technology for the treatment of high-strength wastewater under extreme environmental conditions and it has also been used in combination with other treatment technologies [2].

In general, this process is based on plastic carriers on which biomass attaches and grows. The most knows biofilm carriers look like very small wagon wheels and they are made of high density polyethylene (HDPE) with a density around $0.95 g/cm^3$. They have the shape of small cylinders, with a 4-8 sppokes on the inside of the cylinder and "fins" on the outside. The size of

the carrier varies from hights of 7-15mm and diameters of 10-15mm. The carrier filling fraction (% of reactor volume occupied by the biofilm carriers in the empty basin) is approx. 40–70%. The biofilm, which grows in the internal structures of the carriers, degrades the pollutants dissolved in the wastewater flow. Each biofilm carrier helps increase productivity by providing an active surface that supports microorganisms involved in degradation processes. The lifespan of biofilm carriers (depending on shape, material and usage condition) available on the market varies from 10 - 30 years [14].

As mentioned before, high density polyethylene is one of the most preferred material to produce biocarriers due to its plasticity, density and durability. However, high hydrophobicity and low surface energy ($30 mJ \cdot m^{-2}$) has been reported to limit initial microbial cell attachment in HDPE carriers [15]. Increasing the bacterial attachment rates, hence, reducing mature biofilm formation startup could be address modifying surface plastic carriers through physico-chemical properties (e.g., hydrophobicity and/or positive charges, types or location of superficially functional groups), and biological means. Several types of processes can modify carriers' materials surface characteristics: i) wet chemical oxidation; ii) polymer grafting or blending; iii) heterotrophic growth.

In this paper, we analyzed the influence of cellulose in conservation and enhancing the bacterial adhesion to synthetic artificial materials *(*SAM) based on *HDPE.*

SAM is an organized layer of amphiphilic molecules that are chemisorbed on the surface via terminal thiol groups. Closed-pack ordered structure with functional groups sticking out from the surface are formed as a result of dispersion forces between the long hydrophobic chains [16]. These types of biocarriers were first materials used in MBBR systems and they are made, generally, of polyethylene with a close-to-water density enriched with different types of other substances to help bacterial adhesion. Bacteria adhere onto more hydrophobic and positively charged surfaces compared to negatively and hydrophilic surfaces. Cellulose is a major component in a rigid cell walls of plants and it is a linear polymer with many glucose monosaccharide units. An important feature of cellulose, relatively unusual in the polysaccharide world, is its crystalline structure of cellulose into simple sugars is one of the major problems in biodegradation industry. Although a large number of microorganisms (fungi, bacteria and actinomycetes) are capable of degrading cellulose, only a few of them produce significant quantities of cell-free enzyme fractions capable of complete hydrolysis of cellulose in vitro [17]. There is a possibility that microbial biofilms could also be used as agents of cellulose degradation due to their favorable properties. A biofilm is a complex aggregation of microorganisms, which is characterized by structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substances. The extra-cellular polysaccharides in this matrix have a variety of binding sites for extracellular enzymes and substrate macromolecules, facilitating the extracellular enzymatic reactions [18].

Experimental

Materials

This study was aimed influence bacterial growth of *Escherichia coli* and its adhesion in the presence of four SAMs with different proportions of HDPE, cellulose and talcum (Table 1).

E. coli is primarily known as a commensal colonizing the gastrointestinal tract of human and animals very early in life but some strains being responsible for diseases. Prior to human infection, enteropathogenic *E. coli* can be found in animal reservoirs, contaminated food matrices, food process environments and natural environments from where it reaches the wastewater treatment plants [19]. Therefore, *E. coli* is a common bacterial strain to wastewater treatment plants that can play an important role in the treatment process.

The bacterial growth rate of *E. coli* ATCC 25922 incubated in LSB (Lauryl Sulphate Broth) (Oxoid USA) was analyzed after in one-hour growth at 24-25°C. The colony forming

units (CFU/mL) were quantified by colony count method using Yeast Extract Agar medium (Oxoid, USA). Positive control of LSB with *E. coli* ATCC 25922, negative controls (*Enterococus faecalis* ATCC 29212) and blank control of LSB were analyzed.

Methods

Each SAM (Table 1) was immersed in 100mL *E. coli* ATCC 25922 (74 x 10^3CFU/mL) suspension with Lauryl Sulphate Broth (LSB) and incubated 1hour at 24-25°C. Previous to bacterial incubation assay, SAMs were sterilized at 121℃ in an autoclave (Systec, GmbH) for 15 minutes. The sterilization process was controlled by using biochemical indicators of sterilization efficiency and also by inactivating the biological indicator *Geobacillus stearothermophillus*. After incubation the SAM were removed, washed twice with distilled sterile water then the adherent bacteria were removed with buffer PBS (1X) from SAM and counted on the hemocytometer in presence of the Trypan Blue. The CFUs from remaining supernatant (after SAM removal) of the bacterial suspension were quantified, too.

Table 1. The composition of SAMs used in bacterial adhesion

SAMs	SAMs type	SAMs composition
SAM 1	Cream	95% HDPE + 5% talcum
SAM 2	Yellow	92% HDPE + 5% talcum + 3% cellulose
SAM 3	Red	90% HDPE + 5% talcum + 5% cellulose
SAM 4	Brown	88% HDPE + 5% talcum + 7% cellulose

Results and discussion

Bacterial growth

The optimal growth conditions of the *E. coli* (ATCC 25922) tested in presence of LSB showed a bacterial suspension density increased by 3890CFU/mL in 1 h as it is presented in Figure 1, which means that the bacteria were viable and able to multiply under the given conditions.
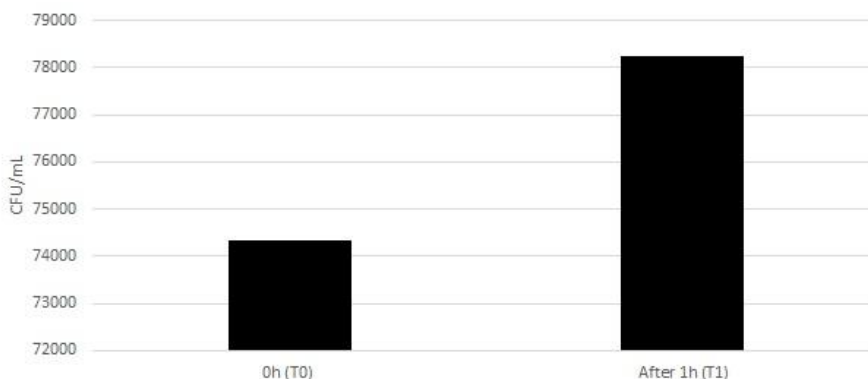

Fig. 1. Bacterial growth. CFUs were measured after 1h incubation and 24-25°C

The bacterial suspension, quantified at 74200CFU/mL, was further used for the bacterial adhesion on SAM assays.

Bacterial adhesion on SAMs

The monitoring of the bacterial adhesion was initiated at 74350CFU/mL bacterial suspension in which various types of SAM were incubated 1h. It could be observed that a bacterial growth without SAM increased the bacterial density (Fig. 1), but in presence of various types of SAM the remained supernatant (SN) bacterial density decreased after SAMs removal (Table 2). The decrease was more and more significant with the increased cellulosic

percent in the SAM composition. The SN after SAM 1 removal (no cellulose) had a bacterial density decreased by 391CFU/mL. SAM 2 (3% cellulose) decreased the bacterial density in the SN by 2085CFU/mL, SAM 3 (5% cellulose) decreased by 2970CFU/mL and SAM 4 (7% cellulose) by 4363CFU/mL.

Decreasing of the bacterial density was directly linked to the % of cellulose in the SAM composition and it could be considered an indirect proof for *E. coli* adhesion on SAM.

Table 2. Monitoring the bacterial adhesion various SAMs, based on their cellulosic composition

LSB + SAM + *E.coli*	T0 [CFU/mL]	T1 [CFU/mL]
SAM 1	74253	73862
SAM 2	74241	72156
SAM 3	74238	71268
SAM 4	74205	69842

A direct proof of the bacterial adhesion on SAMs composed by various percent of cellulose was achieved by counting the bacterial suspensions removed directly from SAMs. The bacterial count was quantified by a hemocytometer in presence of Trypan Blue.

The results showed a contestant increase of bacteria attached to SAMs which is directly linked with the increase of the cellulosic percent from the SAMs composition. The number of bacteria attached to SAM 1 (0% cellulose) slightly increase for bacteria attached to SAMs 2 (3% cellulose) and SAM 3 (5% cellulose), but in the case of SAM 4 (7% cellulose) the increase was more than 20% as it is presented in Figure 2.
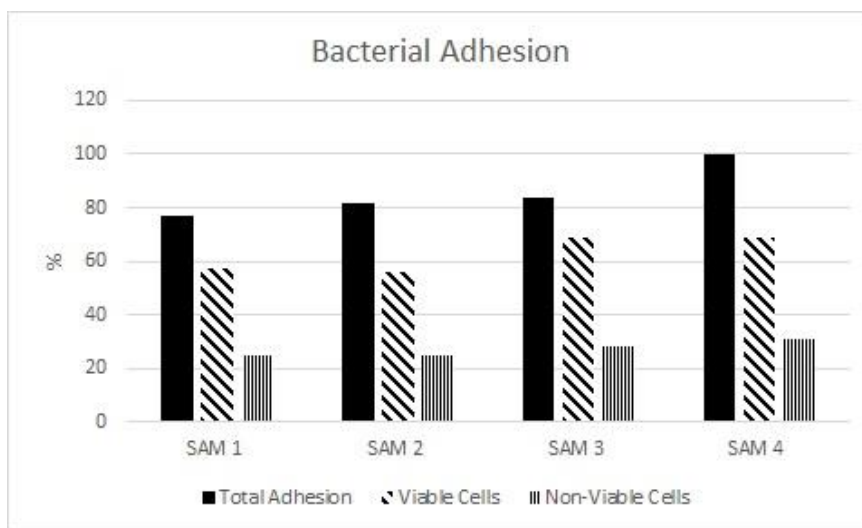

Fig. 2. Bacterial adhesion on SAMs with different proportions of cellulose

It was interesting to observe that the ratio between viable and non-viable bacteria remained, overall, in the same order of magnitude (3:1) regardless of the cellulosic percent. I seemed that the viability was not significantly influenced by the percent of cellulose. On the opposite, the percent of cellulose significantly influenced only the overall bacterial adhesion.

The overall proof of bacterial adhesion on SAMs correlated the indirect proof of bacterial density in the remained SN, measured by CFU/ml, with the direct proof of bacterial adherent on SAMs, measured by microscopy. Results showed that more bacteria adhered to SAM, the less remained in the SN and this rapport was enhanced by the presence of more and more cellulose in the SAMs composition.

Conclusions

Overall, the SAM composition played an essential role in the conserving the bacterial binding capacity and moreover, enhancing the SAMs binding sites. The cellulose was a key structure in enhancing the binding capacity of SAM without altering the bacterial viability. 7% of cellulose in the composition of HDPE and talcum SAM clearly enhanced the bacterial adhesion, preserving their viability.

Acknowledgments

References

[1] C. Bumbac, E. Manea, C. Stoica, A. Banciu, L. Ionescu, V. Badescu, M. Nita-Lazar, *The intertrophic relationship between algae and bacteria from the activated microalgae granules*, **Revista de Chimie, 70**(1), 2019, pp. 319-323.

[2] M. Nita-Lazar, S. Gheorghe, A. Anghelache, A. Banciu, C. Stoica, I. Lucaciu, *Modulation of the bacterial defense mechanisms by various chemical structures*, **Revista de Chimie, 67**(8), 2016, pp. 1454-1457.

[3] M. Nita-Lazar, T. Galaon, A. Banciu, I. Paun, C. Stoica, I. Lucaciu, *Screening of various harmful compounds in a new bacterial biological model*, **Journal of Environmental Protection Ecology, 17**, 2016, pp. 237-247.

[4] F. Chen, J. Ricken, D. Xu, S.V. Wegner, *Bacterial Photolithography: Patterning Escherichia coli Biofilms with High Spatial Control Using Photocleavable Adhesion Molecules*, **Advances Biosystems, 3**(3), 2018, pp. 1-6.

[5] A. Catrangiu, D. Niculescu, I. Lucaciu, C. Chifiriuc, G. Mihaescu, V*irulence factors of Gram-negative bacteria isolated from natural aquatic ecosystems*, **Journal of Environmental Protection Ecology, 16**(1), 2015 pp. 33-39.

[6] C. Stoica, S. Gheorghe, J. Petre, I. Lucaciu, M. Nita-Lazar, *Tools for assessing Danube delta systems with macro invertebrates,* **Environmental Engineering and Management Journal, 13**, 2014, pp. 2243-2252.

[7] C. Stoica, J. Camejo, A. Banciu, M. Nita-Lazar, I. Paun, S. Cristofor, *Water quality of Danube Delta systems: ecological status and prediction using machine-learning algoritms*, **Water Science and Technology, 73**(10), 2016, pp. 2413-2421.

[8] X. Guo, B. Li, R. Zhao, J. Zhang, L. Lin, G. Zhang, R. Li, J. Liu, P. Li, Y. Li, X. Li, *Performance and bacterial community of moving bed biofilm reactors with various biocarriers treating primary wastewater effluent with low organic strength and low C/N ratio,* **Bioresource Technology, 287,** 2019, pp. 1-8.

[9] K. Czaczyk, K. Myszka, *Biosynthesis of Extracellular Polymeric Substances (EPS) and Its Role in Microbial Biofilm Formation,* **Polish Journal of Environmental Studies, 16,** 2007, pp. 799-806.

[10] A. di Base, M.S. Kowalski, T.R. Devlin, J.A. Oleszkiewicz, *Moving bed biofilm reactor technology in municipal wastewater treatment: A review*, **Journal of Environmental Management, 247**, 2019, pp. 849-866.

[11] A. Barwal, R. Chaudhary, *To study the performance of biocarriers in moving bed biofilm reactor (MBBR) technology and kinetics of biofilm for retrofitting the existing aerobic*

treatment systems: a review, **Review in Environmental Science and Bio/Technology, 13,** 2014 pp. 285–299.

[12] H. Ødegaard, B. Rusten, T. Westrum, *A new moving bed biofilm reactor – Applications and results*, **Water Science and Technology, 10-11,** 1994, pp. 157-165.

[13] Y.X. Cui, D. Wu, H.R. Mackey, H.K. Chui, G.H. Chen, *Application of a moving-bed biofilm reactor for sulfur-oxidizing autotrophic denitrification*, **Water Science and Technology, 77**(4), 2018, pp. 1027-1034.

[14] A. Barwal, R. Chaudhary, *To study the performance of biocarriers in moving bed biofilm reactor (MBBR) technology and kinetics of biofilm for retrofitting the existing aerobic treatment systems: A review*, **Environmental Science and Biotechnology, 13,** 2014, pp. 285–299.

[15] A. Terada, A. Yuasa, T. Kushimoto, S. Tsuneda, A. Katakai, M. Tamada, *Bacterial adhesion to and viability on positively charged polymer surfaces*, **Microbiology, 152**(12), 2006, pp. 3575-3583.

[16] Y. Delaviz, J.P. Santerre, D.G. Cvitkovitch, **Infections Resistant Biomaterials, Biomaterials and Medical Devices – Associated Infections,** Elsevier, 2015, pp. 223-254.

[17] C.A. O'Sullivan, P.C. Burnell, W.P. Clarke, L.L. Blackall, *Structure of cellulose degrading bacterial community during anaerobic digestion,* **Biotechnology and Bioengineering, 92**(7), 2005, pp. 871-878.

[18] K.M.D. Gunathilake, R.R. Ratnayake, S.A. Kulasooriya, D.N. Karunaratne, *Evaluation of cellulose degrading efficiency of some fungi and bacteria and their biofilms,* **Journal of the National Science Foundation of Sri Lanka, 41**(2), 2013, pp. 155-163.

[19] V. Ageorges, R. Monteiro, S. Leroy, C.M. Burgess, M. Pizza, F. Chaucheyras-Durand, M. Desvaux, *Molecular determinants of surface colonization in diarrhoeagenic Escherichia coli (DEC): from bacterial adhesion to biofilm formation*, **FEMS Microbiology Reviews**, **44**(3), 2020, pp. 314-350.

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