*BIO*AUTOMATION, 2009, **13** (4), 211-220

Influence of Polymer Substratum onto *Arthrobacter Oxydans 1388* Biofilm Formation

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Summary: Microorganisms attach to surfaces and develop biofilms. Biofilm-associated cells can be differentiated from their suspended counterparts by generation of an extracellular polymeric substance (EPS) matrix, reduced growth rates, and the up- and down- regulation of specific genes. Attachment is a complex process regulated by diverse characteristics of the growth medium, substratum, and cell surface. Biofilms have great importance for public health because of their role in certain infectious diseases and importance in a variety of device-related infections. Biofilms are composed primarily of microbial cells and EPS. EPS may account for 50% to 90% of the total organic carbon of biofilms and can be considered the primary matrix material of the biofilm. EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Extracellular polymeric substances (EPS) are biopolymers of microbial origin in which biofilm microorganisms are embedded. Exopolysaccharides and proteins are one of the main factors in biofilm formation, evidence for microenviromental changes of microbial cells. The aim of this study was to investigate the formation, growth and biofilm characteristics from strain *Arthrobacter oxydans 1388* onto different kinds of polymer matrixes and biochemical research of EPS production. It was compared three different kinds of polymer matrixes and their influence onto biofilm formation. Matrixes were obtained on the base of copolymer of acrylonitrile with acrylamide and mixed with cellulose acetate butyrate. In this case they were with high mechanical stability. The obtained results demonstrated that the most appropriate carrier for biofilm formation is the polymer matrix on the base of copolymer of acrylonitrile with acrylamide and mixed with cellulose acetate butyrate.

Keywords: Biofilms, EPS, Microorganisms, *Arthrobacter oxydans*, Copolymer.

1. INTRODUCTION

Microorganisms attach to surfaces and develop biofilms. Biofilm-associated cells can be differentiated from their suspended counter-parts by generation of an extracellular polymeric substance (EPS) matrix.

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Biofilms have been cited in the literature for a number of years, often being defined as, "cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin" [2, 8]. Whilst this definition of a biofilm is acceptably portrayed as the universally acknowledged biofilm model, slight reclassification has taken place. This occurred in 1995 with the redefinition of biofilms being "matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces" [4].

Biofilms are dense bacterial structures with high growth and conversion rates, therefore, ideal systems to link populations to activities.

Bacteria experience a certain degree of shelter and homeostasis when residing within a biofilm, and one of the key components of this microniche is the surrounding extrapolymeric substance matrix. This matrix is composed of a mixture of components, such as exopolysaccharides, protein, nucleic acids, and other substances. Most bacteria are able to produce polysaccharides, either as wall polysaccharides (capsules) or as extracellular excretions into the surrounding environment [5].

It is most likely that EPS plays various roles in the structure and function of different biofilm communities. Moreover, it is quite possible that EPS plays a different role in similar microbial communities under different environmental conditions [13].

In this study some biochemical methods for investigation of EPS are described. Quantity determination of extracellular polymeric substances, exopolysaccharide and proteins in particular depends on methods of extraction and hydrolysis [11].

The aim of this study is to investigate formation and development of *Arthrobacter oxydans 1388* biofilm on different kind of polymer matrixes. To verify the vitality of the *Arthrobacter oxydans* biofilm grown on the carriers, the dynamics of its formation is studied by biochemical methods.

Arthrobacter oxydans is a genus of bacteria that is commonly found in soil. Also, species are found in extreme environments, such as the arctic sea, under leaking radioactive waste tanks, and in distilled water.

All species in this genus are Gram-positive obligate aerobes that are rods during exponential growth and cocci in their stationary phase.

Due to their metabolic diversity, *Arthrobacter* species have been used in industrial applications and are currently being used in the bioremediation of contaminated groundwater.

Arthrobacter oxydans plays an important role in controlling the poisoning of the earth through toxins that. Recently, it has been discovered that several species of *Arthrobacter* can reduce concentrations of hexavalent chromium, which can cause severe irritations to humans, and they are also known to degrade agricultural pesticides [6].

Arthrobacter oxydans strain is chosen because of its high urease activity and urea degradation in wastes [12], [18]. It shows capability of utilizing hydrocarbons as nutrition and energy source in media with different pH values [14]. It has also been proven that these bacteria can detoxify chromium [1] and high levels of Ni [17], and high efficiency removal of NH_3 and H_2S from waste gases was achieved with such strains inoculated in a biotrickling filter reactor [3].

Immobilization of *Arthrobacter oxydans* cells on a surface of carrier by adhesion and formation of stable biofilm depends on different factors, like nature of surface cell, age of cell culture, kind of polymeric matrix and environment conditions. These parameters have influence on the adhesion of cells. The adhesion method of immobilization is very simple and easy for realization, and applicable for more sensitive microbial cells. Matrixes were obtained on the basis of copolymer of acrylonitrile with acrylamide and mixed with cellulose acetate butyrate.

2. MATERIALS AND METHODS

2.1. Materials

Salts for nutrient medium were obtained from Merck (Germany). Glucose and bovine serum albumin were obtained from Fluka (Switzerland). All other chemicals were of reagent grade or better.

2.2. Methods

2.2.1. Cell culture and biofilms formation

Arthrobacter oxydans strain 1388 from the National collection for industrial and cell cultures in Bulgaria (NBIMCC) were cultured on solid agar medium for 48 hours at 28°C. After this incubation, colonies were picked on and suspended in liquid nutrient medium. The composition of the nutrient medium was according to [16].

The matrixes that were used in the experiments for formation of biofilm were polymeric granules of three different kinds of polymers or mixture of polymers. The obtained matrixes were placed in the cell suspension with nutrient medium and the biofilms were formed by cell adhesion. The binding of cells was carried out at pH 7 at a temperature 28°C under continuous stirring in bath shaker (220rpm). Investigation of formation of biofilm was studied on 18h, 24h, 48h, and 96h. The obtained biofilms were washed up by physiological solution and suspended in the fresh nutrient medium. Exopolysaccharides and protein production of biofilm was investigated.

2.2.2. Analytical procedures

The absorbance of the biomass of free cells and that produced by biofilms were measured at 590 nm with a Perkin-Elmer Lambda 2 spectrophotometer (Germany). Cell growth was also determined by the dry cell weight method [10] after the samples were dried at 105°C. The protein content was measured using a modified Lowry method [9], as described by Raunkjaer et al. [15].

The exopolyssaccharide content was measured using the anthrone method [7], as modified by Raunkjaer et al. [15] to eliminate the effect of a non anthrone-specific colour development.

2.2.3. Preparation of granulated carriers

For biofilm formation there were used polymeric matrixes from three different kinds of polymer or mixture of polymers with granulated shape.

Copolymer of acrylonitrile with acrylamide was dissolved in DMF to a concentration of 10% and was further used for granulating. The granulating procedure was performed by dropping a polymer solution into water: methanol (3:1[v/v]), containing 1% NaCl. The

stability of granules was achieved by dispersing them in distilled water for 30 minutes, followed by heating to 85^0C for 45 minutes. It was prepared in the form of porous granules, 1.5 mm in diameter. The granules were further dried and heated to 70^0C for 3 hours.

The next used polymer carrier was obtained from polymethyl-methacrylate (PMMA), according to the procedure above. The third polymer matrix used for biofilm formation was mixture from copolymer of acrylonitrile with acrylamide and cellulose acetate butyrate in correlation 20:1.

2.2.4. Scanning electron microscopy

The samples with biofilms were dried in a vacuum centrifuge, sputter–coated with gold (JEOL JFS 1200) and examined by scanning electron microscopy (SEM) (JEOL JSM - 5510) at 10 kV.

3. RESULTS AND DISCUSSIONS

The microbial cells were immobilized by adhesion onto three different kinds of polymer matrixes. SEM picture examined the biofilm formation onto the most appropriate carrier.

It showed that during the first 24 hours mainly single cells were attached to the surface. After 24 hours of incubation *Arthrobacter oxydans 1388* cells formed microcolonies representing a monolayer close-packed biofilm with regularly arranged cells.

Fig. 1 shows the *Arthrobacter oxydans* cells immobilized on the surface of polymeric matrix based on copolymer of polyacrylonitrile (PAN) with polyacrylamide (PAA) and mixed with cellulose acetate butyrate.

Fig. 2 presents the kinetics of protein synthesis from the *Arthrobacter oxydans* biofilm formed on the three different kinds of polymer matrixes. In this figure, the protein synthesis from the *Arthrobacter oxydans* biofilm formed on PMMA was increased with the time of incubation. After 24 hours the protein synthesis was decreased. The protein production from the *Arthrobacter oxydans* biofilm formed on PAN+PAA and PAN+PAA+Cellulose shows uniform acceleration of proteins values. The maximal value was reached in 96 h – 24 $mg.ml^{-1}$ for biofilm onto carrier from

PAN+PAA+Cellulose and 11.3 mg.ml^{-1} for carrier from PAN+PAA. The exopolysaccharides syntheses from the *Arthrobacter oxydans* biofilm formed on PMMA and PAN+PAA was increased with the time of incubation, and then the synthesis was decreased sharply.

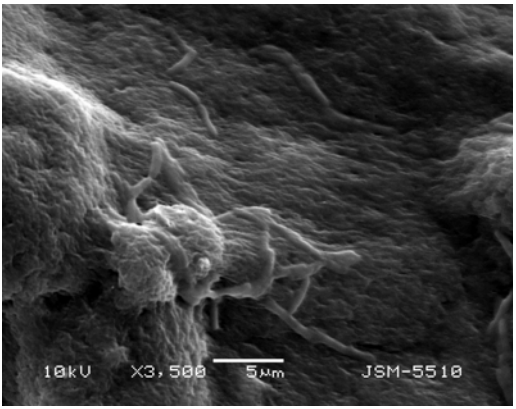

Fig. 1 SEM picture of 24 hours biofilm on matrix based on copolymer of polyacrylonitrile (PAN) with polyacrylamide (PAA) and mixed with cellulose acetate butyrate

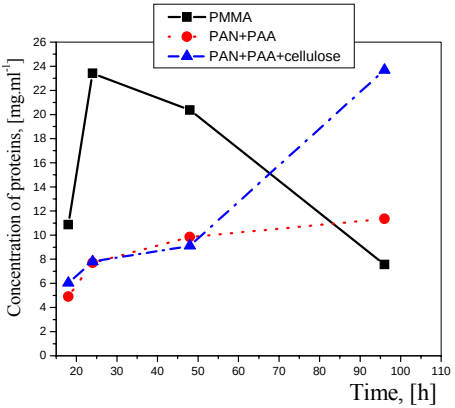

Fig. 2 Kinetics of the protein syntheses from the Arthrobacter oxydans biofilm formed on different three kinds of polymers: PMMA, PAN+PAA, and PAN+PAA+Cellulose

The exopolysaccharides syntheses from the *Arthrobacter oxydans* biofilm formed on PAN+PAA+Cellulose were increased with the time of incubation, and then the synthesis was decreased slowly. The best synthesis of exopolysaccharides was obtained when the *Arthrobacter oxydans* biofilm was formed on PAN+PAA+Cellulose, which is appropriate and more stable for this formation.

Fig. 3 presents the exopolysaccharides syntheses from the *Arthrobacter oxydans* biofilm formed on the different three kinds of polymers or mixture of polymers: PMMA, PAN+PAA, and PAN+PAA+Cellulose.

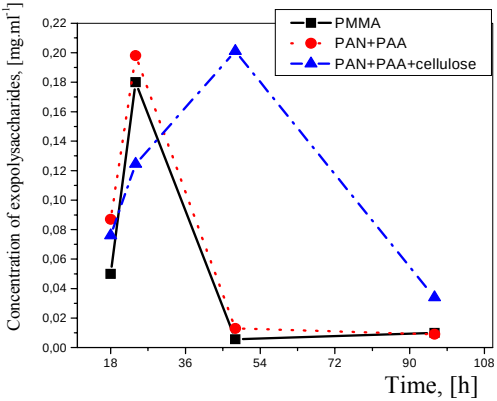

Fig. 3 Kinetics of the synthesis of exopolysaccharides from the Arthrobacter oxydans biofilm formed onto three different kinds of polymers or mixture f polymers
(PMMA, PAN+PAA, PAN+PAA+Cellulose)

Table 1 shows that the fastest immobilization of bacteria in the early hours of study was achieved through adhesion immobilization and therefore the amounts of proteins and exopolysaccharides, produced by *Arthrobacter oxydans*, were higher. Another important conclusion is that the amount of protein and exopolysaccharides of biofilm formed onto carrier from copolymer of acrylonitrile and acrylamide

with cellulose acetate butyrate was higher than the other used polymers.

Table 1. Amount of synthesized proteins and exopolysaccharides at fixed start and end moments of time

Formation of biofilm	Type of polymer	Proteins mg·ml^{-1}	Exopolysac-charides mg·ml^{-1}	Proteins mg·ml^{-1}	Exopolysac-charides mg·ml^{-1}
		18h		96h	
	PAN+ PAA+ Cellulose	6.029	0.076	23.68	0.034
	PAN+ PAA	4.91	0.087	11.4	0.009
	PMMA	10.81	0.05	7.56	0.01

The best polymer matrixes used in this investigation for biofilm formation according to results was carrier from PAN and PAA with cellulose acetate butyrate, because this co-polymer observed relatively smooth surface.

Matrixes from PAN and PAA also seemed to be suitable for the formation of biofilms.

4. CONCLUSIONS

The most suitable polymer matrix for the formation of biofilms was composed of copolymer of acrylonitrile and acrylamide with cellulose acetate butyrate. This co-polymer demonstrated microbial stability and seemed to be suitable for further immobilization processes.

ACKNOWLEDGEMENTS

The present work is funded and supported by project 213, FUND "Scientific Investigations", Ministry of Education and Science, Bulgaria.

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