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Investigation of newly synthesized biocompatible materials as biofilm carriers

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The biodegradation of aniline is of a great concern and has attracted many researchers' attention. Because of its toxic and recalcitrant nature as well as the wide application of aniline containing chemicals, aniline is considered to be an increasing threat both to the environment and to human health.

Microbial transformation and degradation are major mechanisms to eliminate aniline from the environment. Most of the microorganisms found in the nature, industrial and clinical environments are attached to a surface.

The aim of this study is to synthesize new hybrid biocompatible materials, to investigate the obtained matrices for their ability to hold biofilm formation.

It was report about comparison of the formation of biofilms from model gram-negative bacteria *Pseudomonas species 1625* onto different, newly synthesized hybrid carriers. Some kinetic investigations on aniline biodegradation applying obtained biofilms are also discussed.

Key words: biodegradation, aniline, biofilms, carrier.

INTRODUCTION

Aniline is a widely distributed environmental pollutant resulting from the manufacture of dye materials [1] and agricultural chemicals such as herbicides [2]. Because of its toxic and recalcitrant nature and the wide application of aniline containing chemicals, aniline is considered to be an increasing threat both to the environment and to human health. Thus, the fate of aniline in the environments is a great concern. Microbial transformation and degradation are major mechanisms to eliminate aniline from the environment.

Most of the microorganisms found in the nature, industrial and clinical environments are attached to a surface. Separated cells attached to the same surface can "communicate", and thus form a complex structure known as biofilm. Biofilms are complex communities of microorganisms attached to any surface or associated with interfaces. They could be successfully used in a process of bioremediation. This is an innovation technology, which controls the pollutants using biological systems for degradation or biotransformation of different toxic compounds. Taking in consideration the efficiency of the bioremediation by means of different microbial strains the aim of this study is to investigate the creation and application of biofilms for wastewater treatment.

The biodegradation of aniline is of a great concern and has been attracted many researchers' attention. Up to date, it is well recognized that aniline can be efficiently removed by aerobic biological treatment [3–5], and many aniline-biodegradation bacteria such as *Pseudomonas sp*. [6]. Microbial transformation and degradation are major mechanisms to eliminate aniline from the environment. Bacterial species of *Pseudomonas* [7–9], *Rhodococcus* [10], *Frateuria* [11], *Moraxella* [12] and *Nocardia* [13] have been shown to be able of aniline and its derivatives degradation.

Some recent investigations show that the isolated strain, PN1001, a member of the *Pseudomonas species* is capable to do degradation of 93% and 89% of pentylamine and aniline, respectively. Additionally, authors revealed that aniline being

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more toxic demonstrates a more complex degradation pathway [14].

The aim of this study is to synthesize new hybrid biocompatible materials, to investigate the obtained matrices for their ability to hold biofilm formation as well as to synthesize and characterize new three-dimensional co-networks based on pure organic N–acryloylglycine. Additionally herein, we report about comparison of the formation of biofilms from model gram-negative bacteria *Pseudomonas species 1625* onto different, newly synthesized hybrid carriers. Some kinetic investigations on aniline biodegradation applying obtained biofilms are also discussed.

EXPERIMENTS AND EQUIPMENT

Reagents

2,2'-azobis(2-methyl proponitrile) (AIBN) and glycine were provided by Acros Organics. Poly (ethylene glycol) dimethacrylate (PEGDM 550, Mw = 550 g/mol) N,N'-methylenebisacrylamide (BIS), N, N-dimethylacetamide (DMAc) and acryloylchloride were purchased by Sigma-Aldrich. All products were used without any further purification. *Pseudomonas species 1625* microbial strain was purchased by the National collection for industrial and cell cultures (NBIMCC) of Bulgaria. 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.

Cell culture

Pseudomonas species 1625 were growth on solid agar medium for 24 hours at 28 °C. Further colonies were picked up and suspended in liquid nutrient medium at pH 7.0 (14 g/L yeast extract; 15 g/L potassium aspartate; 8 g/L KNO_3; 0.025 g/L $MnSO_4$; 0.060 g/L $FeCl_3.6H_2O$; 0.025 g/L $(NH_4)_6MoO_{24}.4H_2O$) supplemented with 10% glucose. After incubation for 24 h in bath shaker at 28 °C, pH 7.0, the cells were suspended in the same nutrient medium containing different concentration of aniline under the same conditions.

Carriers for biofilm formation

The following three different polymeric matrices were used for biofilm formation during the experiments:

Ti based matrices were synthesized by incorporation of organic polymer (cellulose acetate butyrate (CAB)) and copolymer of polyacrylonitril and acrylamide (Poly (AN-co-AA)) to inorganic network according to [15]. The other two types of polymer membranes are based on N–acryloylglycine (NAGly) – poly (N-acryloylglycine) (PNAGly).

Preparation of NAGly

NAGly was prepared by adapted method of Bentolila et al. [16]. It was synthesized by a Schotten-Baumann reaction in aqueous phase as following: 4.50 g of glycine (60 mmol) were dissolved in 60 mL of 2M solution of potassium hydroxide. The mixture was cooled at 0ºC with a water ice bath for about 10 minutes. 6 mL of acryloyl chloride (73.6 mmol) were added to the mixture drop wise using a dropping funnel. At the end of the reaction (TLC monitoring) the solution was washed with 2×40 ml of diethyl ether and the separated aqueous phase was acidified to pH = 2. The aim product was extracted with 3×40ml ethyl acetate. After drying the organic phase over $MgSO_4$, the residue was concentrated with a rotary evaporator.

Preparation of membrane based on PNAGly

Two different types of membranes were synthesized according to the methodology described above. The overall composition of the networks was varied from 90 to 10 w% of each compound. All investigated co-networks were reported as PNAGly/PEGDM (x/y) or PNAGly/BIS (x/y). The numbers between brackets (x/y) correspond to the PNAGly and cross linker weight proportions, respectively. For example, a co-network obtained from a mixture of 450 mg of NAGly and 50 mg PEGDM was noted PNAGly/PEGDM (90/10). The mixture was stirred and degassed to remove all traces of oxygen (radical inhibitor). Finally, 25 mg of AIBN were added at the last moment to avoid the rapid decomposition of the initiator. The contents of the flask was taken with a pipette and placed between two glass plates separated by a Teflon film and held together by a clamp system to ensure the sealing of the experimental device. The device was placed in an oven and treated according to the following thermal program: 2.5 h at 60 °C to complete polymerization and then one hour at 120 °C to achieve a post-curing. After polymerization, the crosslinked polymer was detached from the device and vacuum dried at 60 °C.

Formation of biofilm

The obtained matrices 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 and temperature 28 °C under continuous stirring in bath shaker (220rpm).

Biofilm formation was studied at 24, 48, 72, 96 and 120 h. Every 24 h the matrixes were washed up by physiological solution and suspended in the fresh nutrient medium.

Methods

Biochemical analyses

The absorbance of the biomass of free cells and this produced by biofilms was measured at 590 nm with a Perkin-Elmer Lambda 2 spectrophotometer (Germany). The renovation of the biofilm was monitored microscopically as well as by means of the turbidity (OD-590) of the effluent. Cell growth of suspended and immobilized cells was also determined as dry cell weight, according to the method described by Mallette [17]. All samples were dried till they reached a constant weight at 105 °C.

The extracellular protein content attach to the matrixes was measured using a modified Lowry method, [18] as described by Raunkjaer et al [19]. The exopolysaccharide content was measured using the anthrone method modified by Raunkjaer et al. [18] to eliminate the effect of a non anthrone-specific color development.

Gas chromatography analysis

In order to detect the biodegradation of aniline by free cells and biofilms from *Pseudomonas species 1625*, samples of 1 ml were taken at a specified hour and were submitted for testing at the Department of Biotechnology, UCTM, Bulgaria using a Shimadzu gas chromatograph GC-2010 with flame ionization detector (FID) and Rtx-5 column. The following temperature gradient was used. Starting with isocratic temperature of 120 °C for 8 min and then increasing to 220 °C with 10/min step. The temperatures of the injector and detector were 305 °C. The carrier gas was helium at 1.7 mL/min.

RESULTS AND DISCUSSION

Initially, we started with investigation of biochemical properties of formed biofilms onto newly synthesized hybrid membranes. The dynamic of proteins and extracellular polysaccharides production by biofilms were studied.

Dynamics of extracellular proteins production from biofilms of Pseudomonas species 1625 formed onto different polymer matrices

After the culture was developed and a biomass was accumulated, polymer carriers were added to the cell suspension for biofilms formation. The kinetic of proteins and polysaccharides produced by biofilms formed on two different types of polymer carriers for 120 hours was tracked and the 24th hour was assumed as initial period for the incubation. The quantity of proteins synthesized from biofilms of *Pseudomonas species 1625* on different types of matrices is shown on Figure 1.

The figure shows that a larger quantity of proteins is produced from biofilms formed on PNAGly/PEGDM matrix. A gradient increase of the quantity of the produced proteins was observed until the 72nd hour of incubation onto two types of biofilms. Further the protein concentration followed by a significant rise to the 96th hour into biofilm formed onto PNAGly/PEGDM matrix. The production again became gradient, reaching 106.87 mg.g^{-1} at the 120th hour.

The concentration of proteins from biofilm formed on a matrix PNAGly/BIS increased proportionally to the incubation time of maturing.

The figure also reveals that considering the produced proteins, the polymer matrix based on PNAGly/PEGDM is a better carrier for biofilm formation.

Dynamics of extracellular polysaccharides production from biofilms of Pseudomonas species 1625 formed onto different polymer matrices

Kinetics of polysaccharide production from the formed biofilms is presented on Figure 2. The figure shows that the quantity of polysaccharides produced from biofilms formed on a matrix of

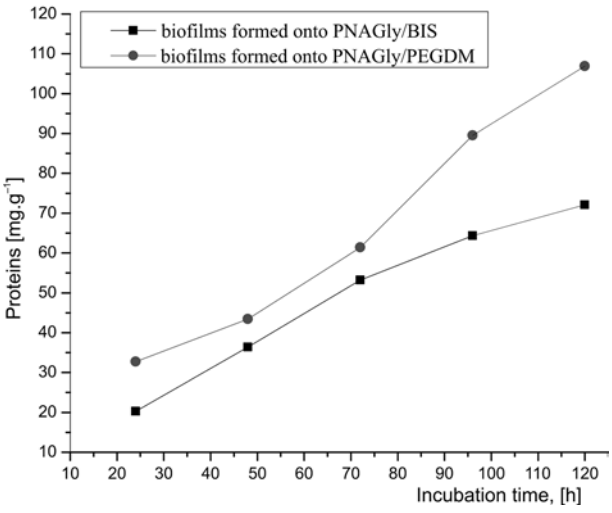

Fig. 1. Kinetic of proteins production from biofilms formed on PNAGly/BIS and PNAGly/PEGDM matrices

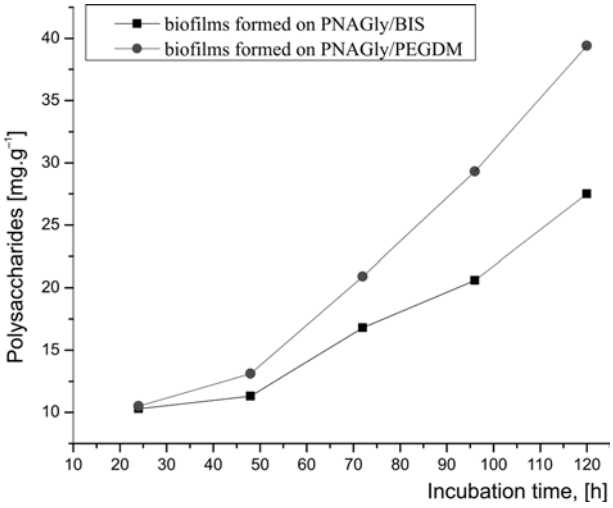

Fig. 2. Kinetics of the polysaccharides production from biofilms formed on PNAGly/BIS matrix and PNAGly/PEGDM matrix

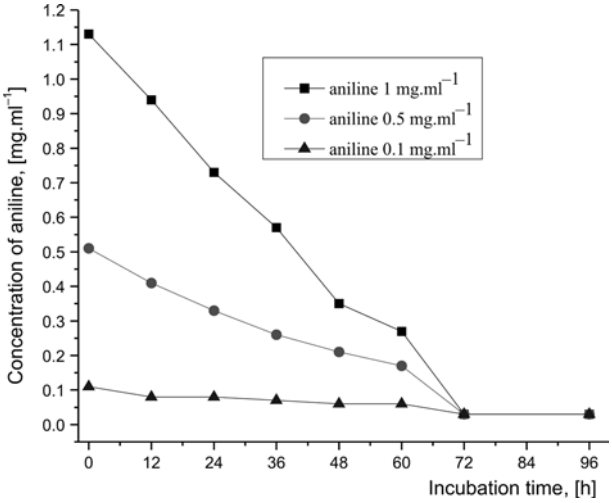

Fig. 3. Dynamics of the aniline degradation by free cells of *Pseudomonas species 1625*

PNAGly/PEGDM is larger. It can be clearly observe that until 48 h of incubation time polysaccharides production of biofilm formed onto PNAGly/BIS membrane is keeping proportional to the incubation time, after that slightly increasing. After 48 h increasing of polysaccharides producing occurring to both biofilms but it is more expressive to biofilm formed onto matrix of PNAGly/PEGDM. The peak about 40 mg.g^{-1} is at 120th hour.

Comparing the proteins and polysaccharides production from biofilms on the two types of carriers we could conclude that the most appropriate matrix for biofilm formation is PNAGly/PEGDM.

Dynamics of the model wastewater purification process of biodegradation of aniline from biofilms formed onto three different types of matrices

In the present study the dynamics of aniline degradation from biofilms formed onto different types of carriers was also investigated. Our study starts with tracking of aniline degradation by free cells of *Pseudomonas species 1625*. Single nutrient source aniline in different concentrations: 0.1 mg.ml^{-1}, 0.5 mg.ml^{-1} and 1 mg.ml^{-1} were used. Samples were taken at every 6 h and they were monitored by gas chromatography.

The results for dynamics of degradation are shown on Figure 3.

Figure 3 shows that decreasing of the concentration of substrate is the best using as an initial concentration 0.5 mg.ml^{-1} and 1 mg.ml^{-1} of aniline. Degradation is proportional to incubation time and after 72 h the aniline is completely biotransformed. Concerning the lowest initial concentration of aniline it is observed a detention and slightly decreasing after 60 h.

On the ground of experimental results mentioned above in this study it was followed biodegradation of aniline by biofilms formed onto three different types of carriers in the initial concentration of 1 mg.ml^{-1}.

On Figure 4 is presented dinamics of aniline degradation by biofilms formed from *Pseudomonas species 1625*.

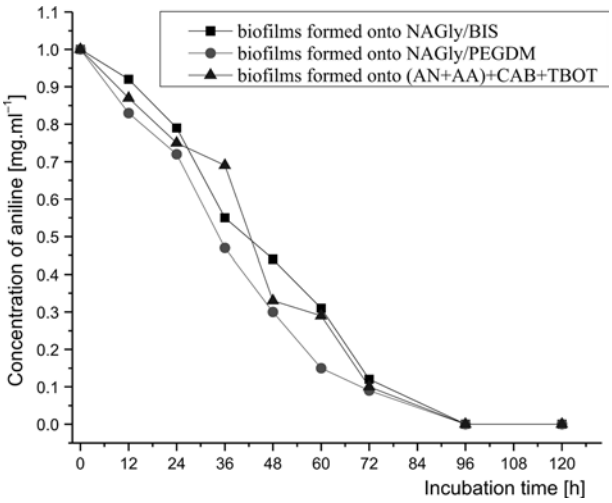

Fig. 4. Dynamics of the aniline degradation in initial concentration of 1 mg.ml^{-1} by biofilms of *Pseudomonas species 1625* formed onto different types of carriers

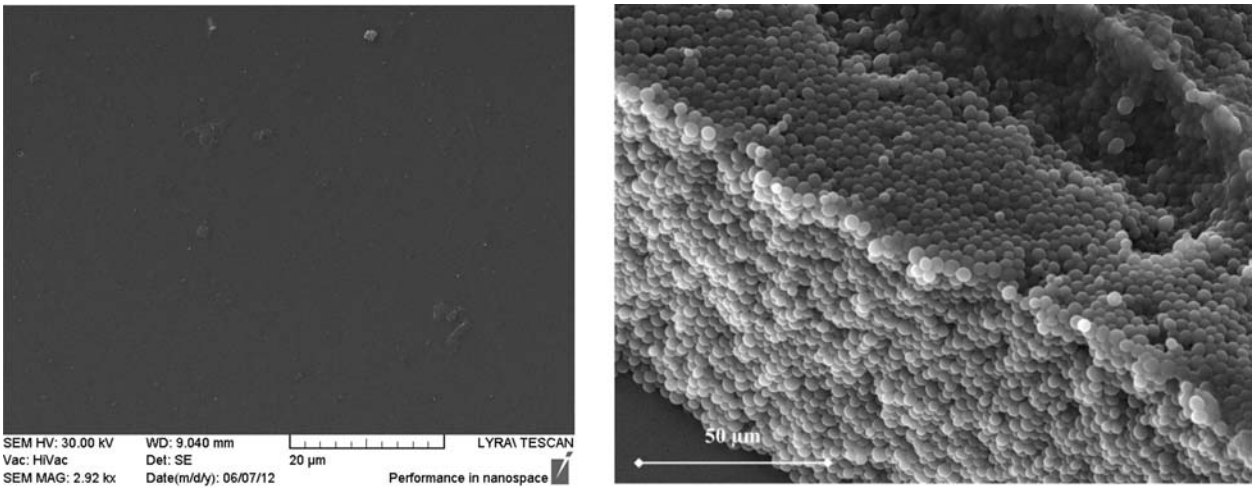

Fig. 5. SEM images of the surfaces of PNAGly/PEGDM sample without (a) and with (b) cells of *Pseudomonas species 1625*

Figure 4 reveals that the application of biofilms is preferable to free cells as it is clearly observed that they are more capable of substrate degradation at initial concentration of 1 mg.ml^{-1}. In addition, it was showed that all three biofilms do completely degradation of aniline after 96 h of incubation time.

In the biofilm formed onto polymer carrier based on (AN+AA)+CAB+TBOT the depletion of aniline is characetrisized with a slightly detention between 24 and 60 h. For biofilms formed onto new hybrid polymer matricies based on PNAGly/BIS and PNAGly/PEGDM is observed that aniline degradation is proportional of the incubation time. From the experimental results is shown that the aniline degradation of biofilm formed onto matrix of PNAGLy/PEGDM is the best expressed.

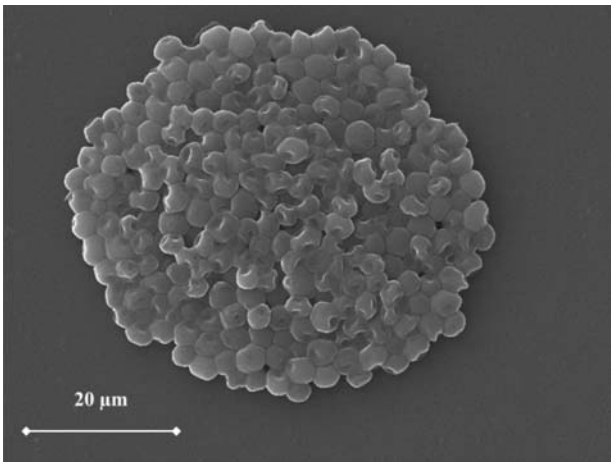

Fig. 6. SEM images of the biofilms formed onto surfaces of PNAGly/BIS

Structure and stability of biofilms

The structure of obtained biofilms was visualized by electronic microscopy. Some of the results are shown in Figure 5 and 6.

SEM images show that *Pseudomonas species 1625* cells are formed a biofilm onto both matrices. But the microscopic analysis clearly presents that the biofilm is thicker onto matrix based on PNAGly/PEGDM.

CONCLUSION

Our study reveals that it is possible to preserve the biological integrity of a living organism (*Pseudomonas species*) in newly obtained matrices. Additionally, the obtained results show that the most appropriate carrier for biofilm formation from the cells of *Pseudomonas species 1625* is the matrix based on NAGly/PEGDM. Finally our results reveal that the matrix obtained by PNAGly/PEGDM is the best according to biofilm formation and aniline biodegradation.

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REFERENCES

1. U. Meyer, J (eds), Microbial degradation of xenobiotics and recalcitrant compounds, Academic, London, 1981.
2. P. C. Kearney, D. D Kaufmann, Herbicides: chemistry, degradation and mode of action, 2nd edn, Marcel Dekker, New York SI, 1975.

3. M. Takeo, T. Fujii, K. Y. Takenaka, *Journal of Fermentation and Bioengineering*, **85**(5), (1998).
4. T. Zhang, J. L. Zhang, S. J. Liu, Z. P. Liu, *Journal of Environmental Sciences*, **20** (6), (2008).
5. C. B. Xiao, J. Ning, H. Yan, X. D. Sun, J. Y. Hu, *Chinese Journal of Chemical Engineering*, **17**, (2009).
6. J. G. Anson, G. Mackinnon, *Applied and Environmental Microbiology*, **48**, (1984).
7. C. Hinteregger, M. Loidl, F. Streichsbier, *Microbiology Letters*, **97**, (1992).
8. R. E. Parales, T. A. Ontl, D. T. Gibson, *J. Ind. Microbiol. Biotechnol.*, **19**, (1997).
9. S. I. Kim, S. H. Leem, J. S. Choi, Y. H. Chung, S. Kim, Y. M. Park, Y. N. Lee, K. S. Ha, *J. Bacteriol.*, **179**, (1997).
10. K. Aoki, K. Ohtsuka, R. Shinke, H. Nishira, *Agric. Biol. Chem.*, **47**, (1983).
11. S. Murakumi, A. Takashima, J. Takemoto, S. Takenaka, R. Shinke, K. Aoki, *Gene*, 1999.
12. J. Zeyer, A. Wasserfallen, K. N. Timmis, *Appl. Environ. Microbiol.*, **50**, (1985).
13. R. Bachofer, F. Lingens, W. Schafer, FEBS Lett, **50**, (1975).
14. Li. Wang, S. Barrington, Jin-Woo Kim, *Journal of Environmental Management*, **83**, (2007).
15. L. Yotova, I. Ivanov, *Appl. Biochem. Biotechnol.*, **87**, (2000).
16. A. Bentolila, I. Vlodavsky, C. Haloun, A. Domb, *J. Polym. Adv. Technol.*, **11**, (2000).
17. M. Mallette, T. R. Noris, D. W. Robinsons, *Methods in Microbiology*, vol. 1 ed, Academic Press, London, New York, 1969.
18. O. H. Lowry, N. L. Rosenbrough, A. L Farr, R. J. Randall, *J. Biol. Chem*., **193**, (1951).
19. K. T. Raunkjær, Jacobsen Hvitved, P. H. Nielsen, *Water Res.*, 1994.

ИЗСЛЕДВАНИЯ ВЪРХУ НОВОСИНТЕЗИРАНИ БИОСЪВМЕСТИМИ МАТЕРИАЛИ КАТО НОСИТЕЛИ ЗА БИОФИЛМИ

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(Резюме)

Биоразграждането на анилина е проблем от голямо значение и привлича вниманието на много изследователи. Съдържанието на анилин в околната среда е заплаха за човешкото здраве, което налага разкриване на нови методи за пречистване. Изследователи докладват за редица случаи на микробна трансформация за разграждане на това токсично съединение. Повечето от микроорганизмите в природата в промишлени и клинични среди са прикрепени към дадена повърхност. Целта на това изследване е да се синтезират нови хибридни биосъвместими материали и да се проучи възможността на получените матрици като носители за формиране на биофилми. В настоящото проучване е разгледано сравнението на биофилми, формирани върху различни матрици от клетки на грам положителните бактерии *Pseudomonas species 1625*, както и възможността на биофилмите да бъдат приложени в процесите на биоразграждане на анилин.