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Comparative study on bacteria colonization onto ceramic beads originated from two Devonian clay deposits in Latvia

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***Abstract*: Ceramic carriers for immobilization of microorganisms are widely used in wastewater treatment, air biofiltration technologies, etc. The aim of this study was to compare seven different types of ceramic beads fabricated from two types of Devonian clay, in terms of their appropriateness for bacteria cell attachment and further use for soil/air cleaning technologies. The effect of different ceramic beads on the microbial growth and biofilm formation was studied for pure culture *Pseudomonas putida* MSCL 650 and for bacteria consortium MDK.EKO-7. The highest CFU number recovered from the bead surfaces after 72h cultivation, was in the sets No. 4, 6, and 7, corresponding to one Liepa red and two Planci clay samples, respectively. Besides, a fluoresceine diacetate (FDA) hydrolysis activity of the attached bacteria served as a criterion of biofilm formation. Statistically significant differences of FDA hydrolysis were shown for both, ceramic beads with biomass and without it. Among ceramic beads without biomass, half-spheres of Liepa red clay beads and Planci clay beads significantly differed from other tested samples. These data could point to the notable differences between physical-chemical properties of these beads, which stimulated abiotic FDA hydrolysis. Among ceramic beads with biomass, FDA hydrolysis activity on the half-sphere beads was significantly ($p<0.05$) higher than that on the surface of the whole sphere beads fabricated from Liepa red clay. SEM micrographs of the bead surface showed uneven distribution of bacteria on the surface. The craters (pores) of ceramic bead seem to be the most appropriate sites for bacteria attachment. Experiments on dehydration of the attached *P. putida* at 22 °C showed a decrease of cell viability down to zero in 16 days. It was concluded that Liepa red clay and Planci clay are appropriate for carrier fabrication. The quality of ceramic carrier is dependent on the technological process of ceramic production, bead composition and conditions for biofilm formation.**

***Keywords*: colonization, bacteria consortium, ceramic beads, enzymatic activity**

I. Introduction

Inert carriers for immobilization of microorganisms are widely used in wastewater treatment, air biofiltration, and soil remediation technologies. The main advantages of the use of immobilized cells in comparison with the suspended ones include retention of higher concentrations of microorganisms, protection of cells against toxic substances and prevention of suspended bacterial biomass in the effluent [1]. Moreover, immobilization of microbial cells provides, in general, high degradation efficiency and good operational stability [2] - [4].

Recently, various ceramic immobilization supports have been tested in the broad spectrum of biotechnological processes. In particular, ceramic porous cubes Vukopor S10 were used as biofilm supports for the continuous acidogenic digestion of olive mill wastewaters to produce volatile fatty acids in anaerobic packed biofilm bed reactor [5]. Biolite® P 3.5 mm beads with the nominal porosity of 37.95% and the average pore size of 0.0664 µm were tested in the laboratory-scale phenol biodegradation process [4]. Ceramic honeycomb support for immobilization of *Burkholderia pickettii* was used for degradation of quinoline in continuous flow operation conditions [6]. An air-lift bioreactor, with a honeycomb-like ceramic column was tested for degradation of 2,4-dichlorophenol by immobilized *Achromobacter* sp. [7]. Capillary and tubular ceramic membranes were tested for spore immobilization of *Phanerochaete chrysosporium*. The highest average biofilm thickness was shown for 3 µm capillary ceramic membranes [8].

An appropriate biofilter media must have large surface area for both adsorption of contaminants and for supporting the microbial growth [9]. It should be mechanically resistant. Besides, the attachment of microorganisms to a surface depends on the microbial structure. The main microbial structure involved in this process is the glycocalyx, which consists of extracellular polysaccharides [9].

The interaction of cells with the ceramic support results in a remarkable increment of the metabolic rate of the resulting adsorbed cells, as reflected by the observed enhancement of their respiratory rate and lower lag periods [10] - [13]. Alterations in cell growth, physiology and metabolic activity may be induced by cell immobilization. It has been generally observed that it is difficult to predict the type and magnitude of metabolic changes possible through immobilization [4], [5], [8], [12], [14].

The aim of this study was to compare eight different types of ceramic beads fabricated from two types of Devonian clay, in terms of their appropriateness for bacteria cell attachment and further use for soil/air cleaning technologies. Comparison of ceramic samples was performed by characterization of their effect on the growing culture of Gram-negative bacteria in liquid medium, as well as an enzymatic activity of bacteria attached onto biofilm.

II. Materials and methods

Ceramic beads tested in this study were prepared at the Institute of Silicate Materials, Riga Technical University, Latvia (Fig. 1). Two types of Devonian clays were used for preparation of ceramic beads, i.e., Liepa red clay and Planci clay.

TABLE 1

PHYSICAL AND CHEMICAL PROPERTIES OF CERAMIC BEADS USED IN THE EXPERIMENT

Sample No.	Composition	Tempe-rature, °C	Apparent density (ρ), g/cm^3	Water uptake, %	pH (H_2O)	Redox potential, mV
1	Liepa red clay + 3% sawdust, extruded (whole spheres)	1175	2.09	n.d.	5.7	59.0
2	Liepa red clay + 3% sawdust, extruded, crushed (half-spheres)	1175	1.56	n.d.	5.9	49.4
3	Liepa red clay + 3% sawdust	1150	1.90	10.89	6.0	39.4
4	Liepa red clay + 3% sawdust, reduced	1150	1.95	7.07	6.0	35.1
5	Liepa red clay + 3% sawdust + clay chamotte	1150	2.15	12.90	6.1	36.6
6	Planci clay + 3% sawdust	1200	n.d.	n.d.	6.1	27.6
7	Planci clay + 3% sawdust	1100	1.95	n.d.	6.1	32.0

*n.d. – not determined

Ceramic beads were prepared by plastic shaping and fired in the laboratory furnace at different temperatures [15]. The main physical and chemical characteristics of ceramic beads are presented in Table 1. The chemical composition of Liepa red clay and Planci clay is summarized in Table 2.

Cultivation conditions and bacteria attachment onto ceramic beads

Experiments were performed with a single culture, i.e., *Pseudomonas putida* MSCL 650, as well as a bacteria consortium MDK-EKO-7, consisting of *Pseudomonas* spp. (two strains) and *Stenotrophomonas maltophilia* (six strains). Attachment experiments were conducted in 50 mL flasks containing 30 mL ceramic beads, 30 mL medium and 0.3 mL inoculum. Flasks were incubated at 28 °C with periodical agitation. Medium composition for bacteria cultivation was as follows, g/L: Na_2HPO_4 – 6.0; KH_2PO_4 – 3.0; NaCl – 0.5; $(COONH_4)_2xH_2O$ – 0.4; molasses – 5; yeast extract – 2. Cell concentration was expressed as colony forming units (CFU) per mL and determined by making serial decimal dilutions and plating on TGA (Sifin, Germany). CFU were counted after 72h plate incubation at 28 °C. The number of CFU/mL on a bead surface was determined as follows: before testing beads were dehydrated in Petri dishes at 22 °C or at 37 °C according to the experiment scheme. After that, beads were crushed and then suspended in sterile water for preparation of serial dilutions and plating.

Enzymatic activity

For fluorescein diacetate (FDA) hydrolysis assay one ceramic bead was placed in a tube with 5 ml 0.06 M phosphate buffer pH 7.6, containing 0.4 mg FDA in 0.2 mL acetone. FDA hydrolysis activity was determined after 60 min incubation at 37 °C, in fourplicate, after adding an aliquote of acetone, centrifuged at 4000 rpm and measured at 490 nm [16].

Fluorescence microscopy

Bacteria were stained with DAPI (Fluka) 1 μg/mL or for viability with ViaGram™ Red$^+$ Bacterial Gram Stain and Viability Kit (Molecular Probes, USA) and watched through a fluorescence microscope (Leica DM 2000, Germany). Images were taken with the help of the camera Leica DFC420 and processed by Image-Pro Express 6.0 software.

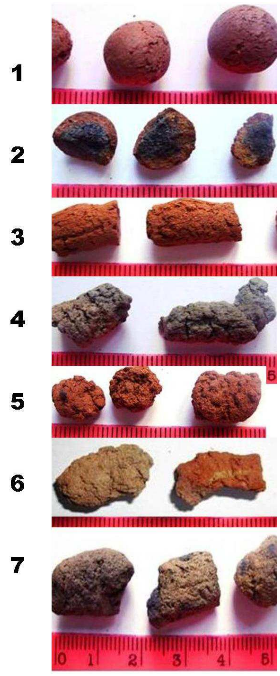

Fig.1. Ceramic beads used in the experiment. Description of the ceramic samples see in Table 1.

TABLE 2

CHEMICAL COMPOSITION OF LIEPA RED CLAY AND PLANCI CLAY

Oxide	Liepa red clay, %	Planci clay, %
SiO_2	62.19	73.56
Al_2O_3	15.45	10.43
CaO	0.83	0.57
MgO	1.32	1.88
Fe_2O_3	7.15	4.06
TiO_2	1.92	0.53
Na_2O	0.09	0.26
K_2O	4.12	3.64
Ignition loss at 1000˚C	4.68	4.60

Scanning electron microscopy (SEM)

Samples were fixed in glutaraldehyde solution (5% final concentration in 0.1 M phosphate buffer, pH 7.2) for 20 h. After washing in water ceramic granules with immobilized cells were incubated in 2% KCl solution for 24 h. The prepared material was dehydrated in acetone of increasing concentrations (40; 60; 80; 90; and 100%, 10 min each), mounted on metal discs. Dried samples were coated with gold in an Eiko Engineering ion coater IB-3 and observed in a scanning microscope Hitachi S-4800 at an acceleration voltage 3.0 kV.

Statistical analysis

The statistical data analysis was performed with the help of the Single factor ANOVA *Excel* software for the significance level α= 0.05.

III. Results and discussion

In this study, a selection of the most appropriate types of ceramic beads for bacteria immobilization was made. growth/viability of bacteria in the presence of ceramic beads was chosen as the main criterion for this selection. In the previous experiments, the clay samples that originated from different Latvia deposits (Prometejs, Liepa, Lode), demonstrated considerable differences towards their effect on the viability and growth of *Pseudomonas putida* MSCL 650. In particular, it was ascertained that the Prometejs ceramic beads possessed the strongest bactericidal effect in the aquatic environment among the tested samples [17]. In the present study, Latvia clay deposits Liepa and Planci served as the source of clay for fabrication of ceramic beads.

Growth of bacteria in the presence of different types of ceramic beads

A typical growth curve for *Pseudomonas putida* MSCL 650 in the presence of the beads No. 7 is shown in Fig. 2. Comparative characteristics of the effect of ceramics on the microbial growth were also given for the bacteria consortium MDK.EKO-7. As shown in Fig. 3, the average CFU concentration of free living cells in culture liquid after 72h incubation was similar for the sets with all tested ceramic beads as well as in the control set without beads. The CFU number in these sets varied within the range from 7.0 x 10^6 to 9.0 x 10^7 CFU/mL (Fig. 3). Fluorescence micrographs of free living cells of bacteria consortium MDK-EKO-7 are shown in Fig. 4.

The three days' cultivation of bacteria consortium in the presence of ceramic beads was expected to result in the formation of a biofilm onto the beads surfaces. The biofilm formation on bead surface was evaluated by three alternative methodical approaches, i.e., (i) counting the number of CFU; (ii) measuring the enzymatic activity of living cells or/and their enzymes; (iii) observation of bead surface with attached cells by SEM.

The number of CFU, which were recovered from the bead surfaces after 72h cultivation, showed that the highest CFU number was in the sets No. 4, 6, and 7, corresponding to one Liepa red and two Planci clay samples, respectively (Fig. 3). Discussing the appropriateness of the plating method for determination of the number of bacteria attached onto the surface, it is important to mention comparative testing conducted within the framework of this study. Before plating, suspension with the beads was treated in three different ways, i.e., (i) 10 min agitation at 100 rpm; (ii) 10 min treatment by ultrasound; (iii) bead crushing by pestle in the mortar. A slightly higher number of CFU was obtained in the samples with the crushed beads and the beads after ultrasonic treatment, as compared to the samples, which were just agitated before plating (results not shown).

Hydrolysis of FDA has been suggested as an appropriate method in integrated bioecosystem studies because the ubiquitous lipase, protease, and esterase enzymes are involved in the hydrolysis of FDA [18]. The results on a microbial FDA hydrolysis activity onto ceramic beads are shown in Fig. 5. The single-factor analysis of variance for ceramic beads with attached cells yielded the Fisher criterion F = 11.31 > F_{cr} = 2.66, where Fcr is the critical value of this criterion.

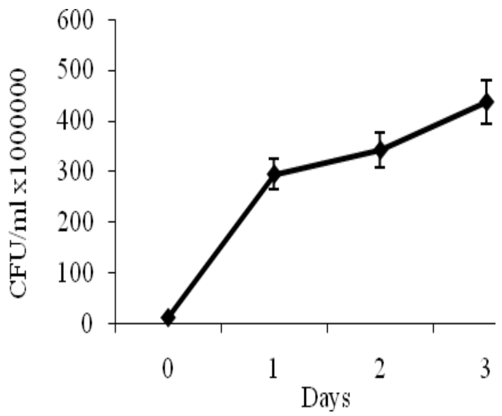

Fig.2. Growth of *Pseudomonas putida* MSCL 650 in the presence of ceramic beads No. 7, at 28 °C. (Description of the ceramic sample see in Fig. 1, Table 1). Error bars represent the standard deviation.

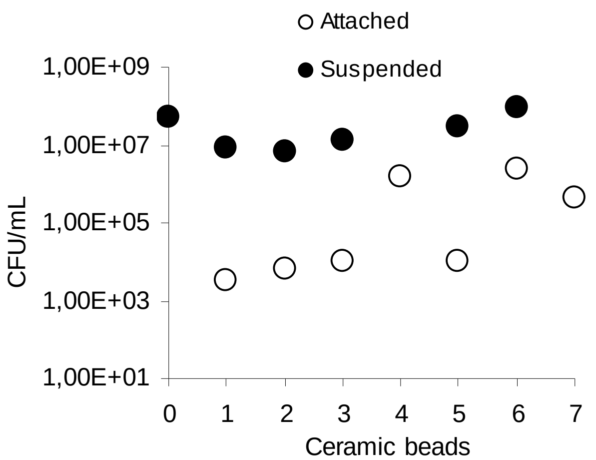

Fig.3. Concentration of bacterial CFU in free (in suspension) and attached state after 72h cultivation of consortium MDK.EKO-7 in liquid broth. 0 – without ceramic beads; 1-7 – different ceramic beads. Ceramic beads with attached cells were dehydrated at 37 °C during 48h before testing. (Description of the ceramic samples see in Fig. 1, Table 1).

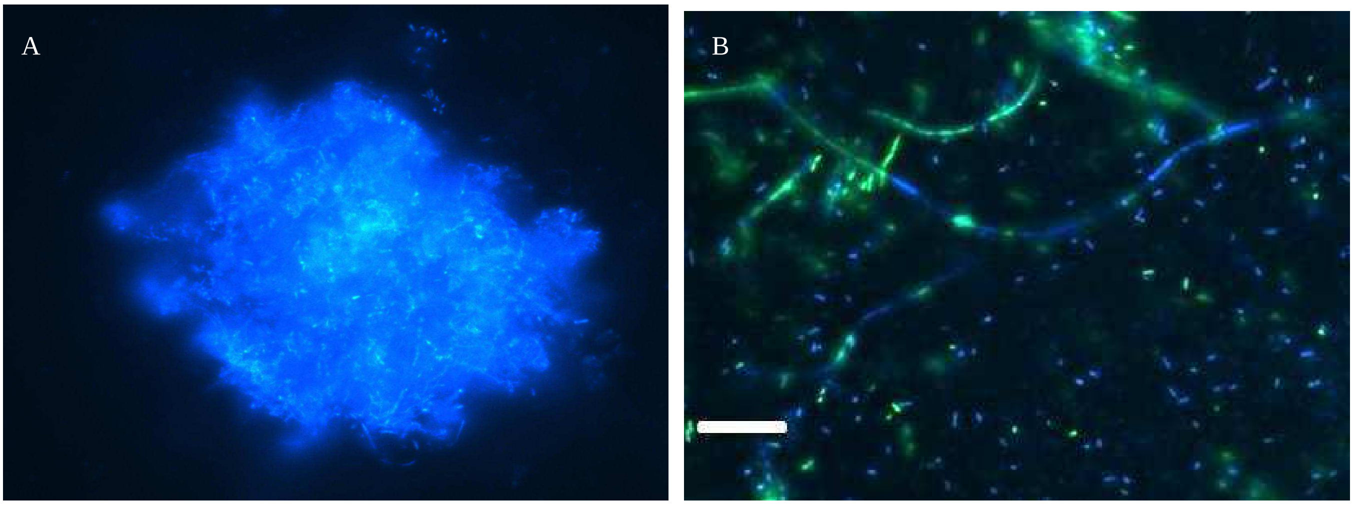

Fig.4 Fluorescence micrographs of bacteria consortium MDK.EKO-7 after 48h growth. A – heterogeneity of suspended bacteria. Live and dead Gram-negative cells fluorescence as blue and green, respectively; B – bacterial colony shows blue fluorescence in the DAPI-stained sample. Bar =10 µm.

Among ceramic beads with biomass, the highest FDA hydrolysis activity was shown to be in the sets No. 2, 4, 5, 6, and 7. No significant differences were detected in this group. The set No. 3 exhibited the lowest enzymatic activity among the tested samples (Fig. 5). In the control variants (ceramic beads without biomass) it was shown that the sets No. 2 and 6 significantly differed from other tested samples.

An additional attention should be paid to the comparison of biofilm formation onto the whole-sphere and half-sphere beads (Sets No. 1 and 2, respectively). In this experiment, the microbial activity on the inner surface of the half-sphere beads was significantly ($F=11.95>F_{0.05;1.16}=4.49$) higher than that on the outer surface of the whole sphere beads (Fig. 5).

Topography of the surface plays an important role in the process of bacteria attachment. The surface properties, in particular, porosity, was supposed to be different on the outer and inner surfaces of the ceramic beads. These differences were observed on the SEM micrographs (Fig. 6). As shown in Fig. 6, bacteria cells are distributed unevenly on the surface. The craters (pores) of a ceramic bead seem to be the most appropriate sites for bacteria attachment.

Survival of Pseudomonas putida attached onto ceramic beads under ambient conditions during storage

The process of immobilization of microorganisms onto a carrier can be performed either, (i) directly in the bioreactor/ biofiltration column with immediate launch of the target biotechnological process or (ii) in a special bioreactor for immobilized biomass preparation „in advance", with further storage, transportation etc. In this respect, great importance is given to viability of the attached cells during storage, in particular, upon slow dehydration at ambient temperature. The results mentioned above, represent the data on bacteria activity after 48h dehydration at 37 °C (Fig. 3, 5). As was previously reported by [19] - [21], the microbial cell viability during dehydration-rehydration process depends on many factors, including temperature. Conversely, spontaneous air-drying of a carrier with immobilized cells may occur at different technological stages. For instance, a sudden short-period or continuous dehydration of cells takes place in a biotrickling biofilter due to uneven distribution of liquid phase in the column. In this respect, the data on cell viability during dehydration at ambient temperatures could provide additional information for further application of the immobilized biomass in biotechnological processes. As shown in Fig. 7, viability of the *P. putida* attached onto ceramic beads No. 7 was decreased down to zero during 16 days of dehydration at 22 °C. Further experiments are needed for more detailed study on viability and activity of the attached bacteria under various conditions.

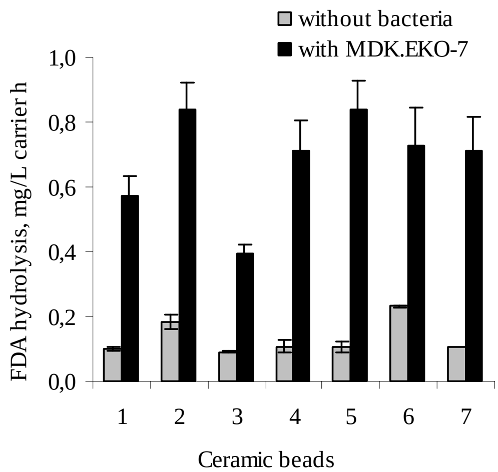

Fig.5. FDA hydrolysis activity of bacteria consortium MDK.EKO-7 attached onto the surface of ceramic beads. 1-8 – ceramic beads (Description see in Fig. 1, Table 1). Ceramic beads with attached cells were dehydrated at 37 °C during 48h before testing. Error bars represent the standard deviation.

Comparative characteristics of ceramic beads originated from Liepa red clay and Planci clay

As shown in our previous experiments, the physical-chemical characteristics of clay notably influence the microbial behaviour towards ceramic surface [17]. In this study, ceramic beads were fabricated from the clay of two Latvia deposits, i.e., Liepa and Planci. As was shown in Fig. 5, half-spheres of Liepa red clay beads and Planci clay beads (sets No. 2 and 6, resp.) without biomass significantly differed from other tested samples by abiotic FDA hydrolysis activity.

Typical porosity and pore distribution for these beads is shown in Fig. 8. The porosity of half-spheres of Liepa red clay bead (set No. 2) (Fig. 8,A) is larger compared to the porosity of granules produced from Planci clay (set No. 6). The difference in porosity is about 10%. The pore size distribution in some ceramic beads (Fig. 8,B) is marked by a slight difference in the region 1 – 0.2 μm of pore diameter. At the same time specific surface area of half-sphere ceramic bead is larger (4.34 m^2/g) in comparison to specific surface area of ceramic bead produced from Planci clay (3.80 m^2/g). These results verify the better biofilm formation and the highest FDA hydrolysis activity of the half-sphere ceramic bead.

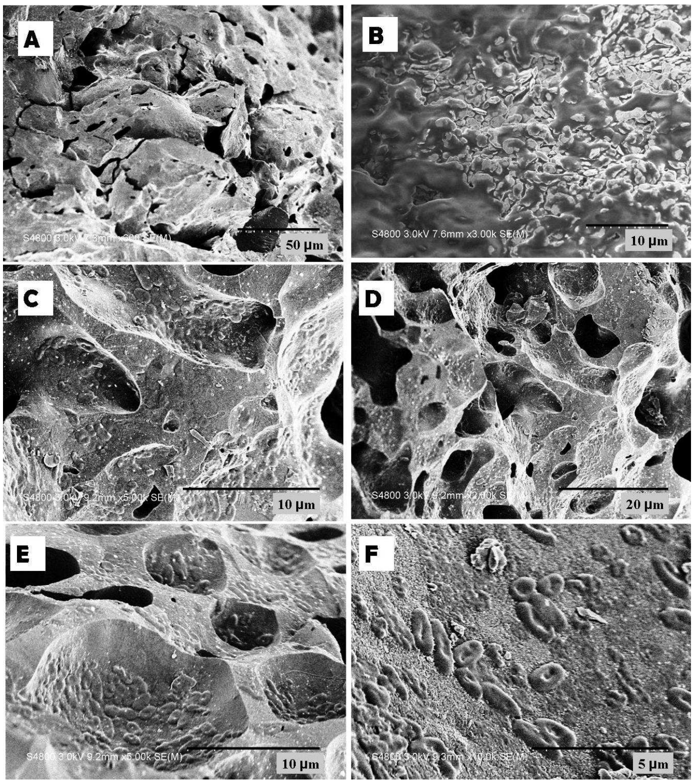

Fig.6. SEM of ceramic beads No. 1 (A, B) and No. 2 (C-F) with attached bacteria consortium MDK.EKO-7.

IV. Conclusions

To summarize the results obtained in this study, the following conclusions are made:

1. The highest CFU number recovered from bead surfaces after 72h cultivation of bacteria consortium MDK.EKO-7, was in sets No. 4, 6, and 7, corresponding to one Liepa red and two Planci clay samples, respectively.
2. Among ceramic beads with biomass, FDA hydrolysis activity on the half-sphere beads was significantly ($F=11.95>F_{0.05;1.16}=4.49$) higher than that on the surface of the whole sphere beads fabricated from Liepa red clay, i.e., sets No. 1 and 2, respectively.
3. Among ceramic beads without biomass, sets No. 2 and 6, corresponding to half-spheres of Liepa red clay beads and Planci clay beads, significantly differed from other tested samples. These data could point to the notable differences between physical-chemical properties of these beads, which stimulated abiotic FDA hydrolysis.
4. The ceramic beads produced from Liepa red clay by addition of chamotte to this clay at the ratio 1:1 (Set No. 5) proved to be an appropriate carrier for bacteria consortium MDK-EKO-7.
5. Experiments on dehydration of the attached *P. putida* at 22 °C showed a decrease of cell viability down to zero in 16 days.
6. Liepa red clay and Planci clay were found to be appropriate for carrier fabrication. The quality of ceramic carrier is dependent on the technological process of ceramic production, bead composition and conditions for biofilm formation. Further experiments are needed for understanding the mechanisms of bacteria attachment onto ceramic beads.

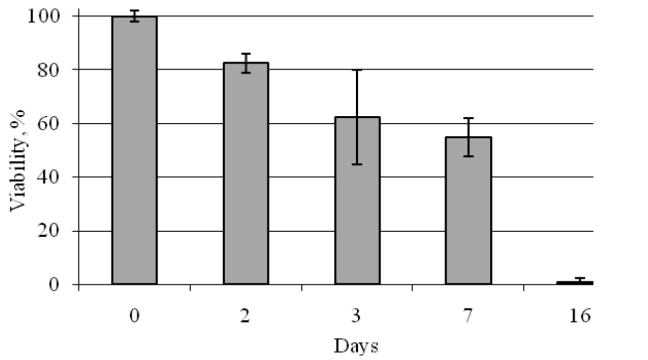

Fig.7. Survival of *Pseudomonas putida* attached onto ceramic beads No. 7, during dehydration at 22 °C. (Description of the ceramic sample see in Fig. 1, Table 1). Error bars represent the standard deviation.

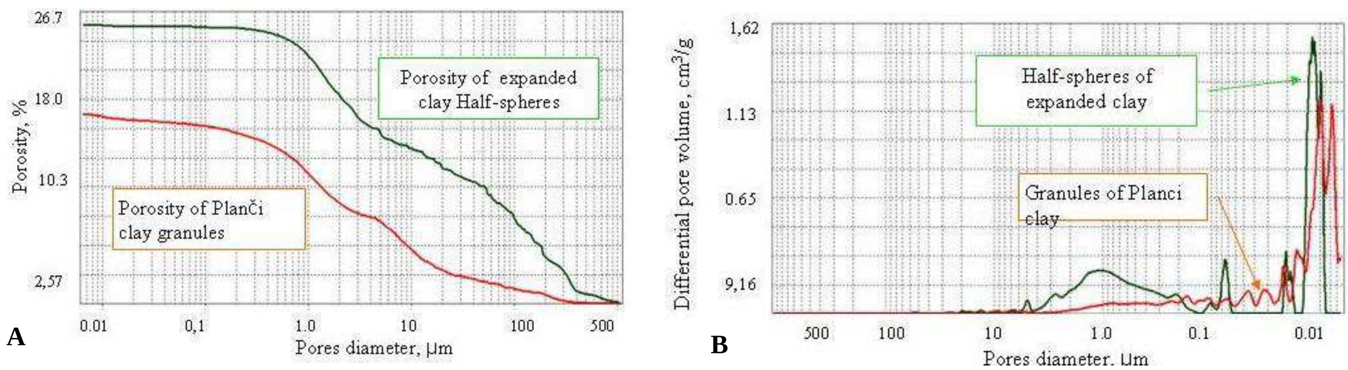

Fig.8. Comparative characteristics of the half-sphere ceramic bead originated from Liepa red clay (set No. 2) and ceramic bead originated from Planci clay (set No. 6) determined by mercury porosimetry. A – porosity, B – pores size distribution.

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Olga Muter, Katrīna Potapova, Vizma Nikolajeva, Zaiga Petriņa, Tatjana Griba, Aloizijs Patmalnieks, Ruta Švinka, Visvaldis Švinka. Baktēriju kolonizācija uz keramikas granulām: divu Latvijas devona māla atradņu salīdzinošā testēšana

Keramikas materiālus mikroorganismu imobilizācijai plaši izmanto notekūdeņu attīrīšanas iekārtās, gaisa biofiltrācijā u.c. tehnoloģijās. Šī pētījuma mērķis bija salīdzināt septiņus keramikas granulu paraugus, kuri sastāv no diviem devona māla veidiem, pēc to piemērotības

izmantošanai mikroorganismu imobilizācijai. Dažādu keramikas granulu ietekme uz mikroorganismu augšanu un bioplēves veidošanu tika pētīta ar tīrkultūru *Pseudomonas putida* MSCL 650 un baktēriju konsorciju MDK.EKO-7. Vislielākais kolonijas veidojošo vienību (KVV) skaits, kuru ieguva no granulu virsmas pēc 72 h kultivēšanas, bija variantos Nr. 4, 6 un 7, kuri atbilst attiecīgi vienam Liepas sarkano mālu paraugam un diviem Planču mālu paraugiem. Kā papildus rādītāju bioplēves veidošanās procesa izpētē izmantoja fluoresceīna diacetāta (FDA) hidrolīzes aktivitāti. Statistiski būtiskas atšķirības FDA hidrolīzes aktivitātē bija konstatētas gan starp sterilo granulu paraugiem, gan starp granulu paraugiem ar bioplēvi. Sterilo granulu paraugi Nr. 2 un 6, kuri atbilst attiecīgi granulu puslodēm no Liepas sarkanajiem māliem un granulām no Planču māliem, ievērojami atšķīrās no pārējiem variantiem. Iegūtie rezultāti var liecināt par granulu fizikāli ķīmiskajām atšķirībām, kuras ietekmē abiotisku FDA hidrolīzi. Salīdzinot granulu paraugus ar bioplēvi Nr. 1 un 2, kuri atbilst attiecīgi Liepas sarkano mālu lodēm un puslodēm, FDA hidrolīzes aktivitāte bija būtiski ($F=11,95>F_{0.05;1;16}=4,49$) lielāka paraugam Nr. 2, t.i., uz pusložu virsmas. Ar skenējošo mikroskopiju bija pierādīts nevienmērīgs šūnu sadalījums uz granulu virsmas, vairums baktēriju atradās keramikas porās. Piestiprināto *P. putida* šūnu dehidratācija 22 °C temperatūrā 16 dienu laikā samazināja šūnu dzīvotspēju līdz 0%. No iegūtajiem rezultātiem secināts, ka Liepas sarkanie un Planču māli ir piemērots izejmateriāls mikroorganismu nesēja izgatavošanai. Keramikas nesēja kvalitāte lielā mērā ir atkarīga no keramikas granulu ražošanas tehnoloģijas, piedevu sastāva un bioplēves veidošanās apstākļiem.

Ольга Мутер, Катрина Потапова, Визма Николаева, Зайга Петриня, Татьяна Гриб, Алоизийс Патмалниекс, Рута Швинка, Висвалдис Швинка. Колонизация бактерий на поверхности керамических гранул: сравнительная оценка двух месторождений глины девонского периода в Латвии

Керамические материалы широко используются в технологиях по очистке сточных вод, биофильтрации воздуха и др. Целью данного исследования было сравнить семь образцов керамических гранул, состоящих из глины двух месторождений девонского периода, - по их пригодности в качестве носителя для иммобилизации микроорганизмов. Влияние различных видов керамических гранул на рост жидкой культуры и формирование биоплёнки оценивали в экспериментах с чистой культурой *Pseudomonas putida* MSCL 650 и консорциумом бактерий MDK.EKO-7. Наибольшее число колонии образующих единиц (КОЕ) с поверхности гранул после 72ч культивирования было выявлено в вариантах 4, 6 и 7, которые соответствуют образцу гранул, изготовленных из глины Лиепа красная и двум образцам, изготовленным из глины Планчи. В качестве критерия оценки формирования биоплёнки, использовали также активность гидролиза флуоресцеина диацетата (ФДА). Статистически значимые отличия были выявлены как в группе со стерильными гранулами, так и среди гранул с биоплёнкой на поверхности. Стерильные гранулы 2 и 6, соответствующие полусферам, изготовленным из красной глины Лиепа и целостным гранулам из глины Планчи, значительно отличались от остальных вариантов гранул. Полученные результаты указывают на различия в физико-химических свойствах, которые влияют на активность абиотического гидролиза ФДА. Среди образцов гранул с биоплёнкой на поверхности, важно отметить отличия в активности гидролиза ФДА между целостными гранулами и механически разделёнными на полусферы. Так, гранулы идентичного состава (красная глина Лиепа), в виде целостных гранул и полусфер, продемонстрировали значительные ($F=11,95>F_{0.05;1;16}=4,49$) различия в активности гидролиза ФДА в пользу последних. Методом сканирующей микроскопии показано неравномерное распределение клеток на поверхности гранулы с наибольшей их концентрациях в порах. Дегидратация прикреплённых к поверхности гранул клеток *P. putida* при температуре 22 °C в течение 16 дней привела к потере жизнеспособности клеток. Глина месторождения Лиепа и Планчи является приемлемым исходным материалом для изготовления носителя. Качество керамического носителя зависит, главным образом, от технологии производства гранул, их состава, а также условий формирования биоплёнки.