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Studying formation of *Pseudomonas aeruginosa* biofilms grown under different cultivation conditions

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SUMMARY

The purpose of the present study is to assess how cultivation conditions influence growth and formation of *Pseudomonas aeruginosa* biofilms. The topic is of great importance due to high incidence of *P. aeruginosa*-caused infections and *P. aeruginosa* resistance associated with its ability to form biofilms. The paper analyzes factors that influence biofilm formation, i.e.: growth phase used for inoculation (log, stationary), volume of the growth medium (0.2 and 1.0 ml) and concentration of nutrients (liquid nutrient media diluted to concentrations of 50; 25; 12.5 and 6%) in the cultivation volume. As the research demonstrates, all these factors influence biofilm formation; and a *P. aeruginosa* growth phase before inoculation is a determining factor in the biofilm formation. When *P. aeruginosa* is inoculated at a stationary phase, biofilm formation shows non-linear dependence on concentration of nutrients and on their total amount in the cultivation volume. The linear dependence of biofilm formation on concentration of nutrients in the culture medium is more pronounced, when *P. aeruginosa* is inoculated at a log phase. The study shows that lower concentrations of nutrient media components lead to more noticeable differences in biofilm formation, and such differences are statistically significant. Two-fold dilution of the liquid nutrient medium does not affect the intensity of biofilm formation; however, a 4 to 8-fold decrease in concentration of nutrients in 0.2 ml of cultivation volume inhibited the biofilms formation. In 1.0 ml of the culture medium, the biofilm forms evenly, and in 0.2 ml of 4–8-fold dilution of nutrient medium it grows slower. The slow growth rate is statistically significant. The cultivation volume is also of great importance. For example, cultures grown in 0.2 ml of nutrient medium at different concentrations of nutrients formed fewer biofilms than microorganisms cultivated in 1.0 ml. At the same time, when inoculating *P. aeruginosa* both at log and stationary growth phases, biofilm formation is more pronounced in wells containing more cultivation volume.

Keywords: biofilms, bacteria, *P. aeruginosa*, stationary growth phase, log growth phase

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Изучение формирования биопленок культурой *Pseudomonas aeruginosa* при различных режимах культивирования

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РЕЗЮМЕ

Настоящее исследование посвящено изучению влияния условий культивирования на рост и формирование биопленок культурой *Pseudomonas aeruginosa*. В связи с высокой частотой встречаемости инфекционных заболеваний, вызванных *P. aeruginosa*, а также устойчивостью синегнойной палочки, в особенности из-за способности образовывать биопленки, данная тема не теряет актуальности. В работе проанализировали влияние на феномен биопленкообразования таких характеристик, как используемая для посева фаза роста культуры (логарифмическая, стационарная), объем среды для выращивания (0,2 и 1,0 мл) и концентрация питательных веществ (жидкие питательные среды, разведенные до концентраций 50; 25; 12,5 и 6%) в объеме культивирования. Проведенные исследования показали, что на образование биопленок оказывает влияние совокупность всех перечисленных параметров. Установлено, что определяющим фактором в формировании биопленок являлась фаза роста бактерий, в которой функционировала культура синегнойной палочки перед инокуляцией. При посеве *P. aeruginosa*, пребывающей в стационарной фазе роста, образование биопленок нелинейно зависело от концентрации питательных веществ и общего их количества в объеме культивирования. Линейная зависимость образования биопленок от концентрации питательных веществ в среде культивирования была более выражена при посеве *P. aeruginosa*, находящейся в фазе логарифмического роста. Установлено, что при меньших концентрациях компонентов питательных сред различия в образовании биопленок были более заметны и имели статистическую значимость. Разбавление жидкой питательной среды в 2 раза не влияло на интенсивность формирования пленки, в то время как 4–8-кратное снижение концентрации питательных веществ в объеме культивирования 0,2 мл ингибировало образование биопленок. В объеме среды для культивирования, равном 1,0 мл, формирование биопленок было равномерным, а в объеме 0,2 мл статистически значимо снижалось при разведении питательной среды в 4 и 8 раз. Объем культивирования также имеет важное значение: так, выращенные в 0,2 мл питательной среды культуры при разных концентрациях питательных веществ формировали меньшее количество биопленок, чем микроорганизмы, культивируемые в объеме 1,0 мл. При этом при посеве *P. aeruginosa*, находящихся как в логарифмической, так и стационарной фазах роста, более выраженным было образование биопленок в лунках с большим объемом культивирования.

Ключевые слова: биопленки, бактерии, *P. aeruginosa*, синегнойная палочка, фаза стационарного роста, фаза логарифмического роста

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INTRODUCTION

The ability to form biofilms may be called one of the most important factors that contributes to persistence and protection of microorganisms from the immune response of the macroorganism [1–3]. A biofilm can be defined as a microbial community attached either to the substrate surface or to each other, surrounded by an exopolymer matrix which is the main structural component of the biofilm. Bacterial phenotype observed in the biofilm is modified compared to single, planktonic cells, the growth and expression parameters of specific genes are transformed [4–7].

The bacterial film is a living, constantly updating community of one or several microorganisms, while the surrounding matrix protects them from adverse environmental impact and serves as one of the factors of intercellular communication. The matrix properties determine the relationship between intracellular community and external environment [3, 6, 8, 9].

More than 95% of all microorganisms are found in natural ecosystems in the form of specifically organized biofilms [5]. *Pseudomonas aeruginosa* (blue pus bacillus, *P. aeruginosa*) is one of the microorganisms capable of forming biofilms. It is a ubiquitous infectious agent, which causes a number of opportunistic diseases. *P. aeruginosa* is detected in 20% of septicemia cases, in 70% of mucoviscidosis cases (in sputum), in up to 70% of hospital-acquired pneumonia cases, in 28% of intra-abdominal infection cases. Totally, it accounts for 20–30% among other etiological agents of hospital-acquired infections [10].

P. aeruginosa is characterized by high resistance to antiseptic substances and disinfectants. The microorganism has a wide range of pathogenicity factors, high epidemic

potential and unique adaptive properties, and is capable of reducing effectiveness of the body's immune response [2, 11–14].

The bacteria in biofilms synthesize an alginate (mucoid exopolysaccharide) and form an exopolymeric alginate matrix. Alginate-producing strains are usually detected in chronic infections, for example, the ones associated with mucoviscidosis. The bacterial biofilm also saves the microorganism from body's natural resistance (lymphocytes, phagocytes, natural movements of the ciliated epithelium in the respiratory tract, antibodies, etc.). The role of quorum sensing systems has also been proven in the *P. aeruginosa* biofilm formation [1, 2, 14, 15].

The importance of the study is explained by the fact that *P. aeruginosa* is an extremely frequent pathogen that plays a key role in emergence of many difficult-to-treat infectious diseases, in particular due to its ability to form biofilms.

There are current scientific studies showing correlation between the growth of plankton culture and biofilm formation, however, this correlation is not absolute [2].

A novelty value of this research consists in studying biofilm formation under different conditions of *P. aeruginosa* cultivation, in particular, depending on the concentration of nutrients in the cultivation volume, as well as the culture growth phase used for inoculation.

The decisive factors in biofilm formation during microbiological experiments are: cultural, enzymatic properties, etc. of the studied microbiota; cultivation conditions (temperature, medium composition, concentration of nutrients, etc.); the material on the surface of which the biofilm will be formed, and much more.

The purpose of this research was to study peculiarities of biofilms formed by *P. aeruginosa* bacterial culture under various cultivation conditions.

MATERIALS AND METHODS

Bacteria. P. aeruginosa ATCC 9027 strain was used as an example to analyze how cultivation conditions influence biofilm growth , the strain was taken from the Museum of the Molecular Biology Sector of the Institute of Experimental Veterinary Medicine of Siberia and the Far East, SFSCA RAS.

Cultivation of P. aeruginosa and bacterial biofilms. *P. aeruginosa* ATCC 9027 culture was grown in LB-Luria liquid nutrient medium (0.5 g/L NaCl; HiMedia) after a preliminary series of passages at room temperature in an incubator shaker for 6 and 24 hours. The broth culture was re-inoculated every 24 hours.

Studying how the growth phase of P. aeruginosa broth culture, volume of the nutrient medium in the well and concentration of nutrient components influence biofilm formation. To assess how *P. aeruginosa* growth phase impacts biofilm formation, 6- and 24-hour culture was inoculated into the nutrient medium, i.e. at log and stationary growth phases (which passed a series of passages in this cultivation phase). Then, *P. aeruginosa* broth suspension was diluted to a value of 0.4 (according to the McFarland standard) in the proportion of 100 µl of culture to 10 ml of nutrient medium. The inoculum was prepared by introducing colonies of *P. aeruginosa* ATCC 9027 strain into sterile saline solution, then bringing the density of the microbial suspension up to the specified concentration.

In order to assess, how volume and concentration of nutrients mutually influence the intensity of *P. aeruginosa* biofilm formation, liquid culture media LB-Luria (0.5 g/L NaCl) and Schaedler (HiMedia) used for cultivation were diluted with saline solution to the concentration of 50; 25; 12.5; 6% and introduced into the wells of flat-bottomed polystyrene plates in volumes of 0.2 and 1.0 ml (in four repetitions). Next, a V-shaped-bottom microplate was immersed into the plate wells, and the *P. aeruginosa* broth culture was inoculated. The inoculations were incubated at a temperature of (25.0 ± 0.5) °C for 18 hours.

LB-Luria and Schaedler (HiMedia) nutrient media were used as a negative control without addition of the microbial inoculum; growth of *P. aeruginosa* was controlled in six repetitions.

Biofilm staining method. In order to assess the growth, the biofilms were stained with gentian violet (crystal violet), a dye that binds to cells and the biofilm matrix, according to the existing method [16] in its modified form: after cultivation is completed, the bacteria, that had not attached to the wells surface were carefully washed away three times with deionized water. The biofilms that formed in the microplate wells were dried at room temperature for 2 hours, fixed with alcohol for 40 minutes and stained with 0.05% solution of gentian violet for 40 minutes. The unbound dye was washed away three times with 0.01 M phosphate-buffer saline with pH 7.2 (3 minutes per one wash). Then a microplate with biofilms was immersed into the wells of a polystyrene flat-bottomed microplate containing 200 µl of 96% ethyl alcohol to elute the unbound dye. The quantitative assessment of the formed biofilms was carried out by measuring optical density with a plate spectrophotometer reader Tecan Sunrise (Tecan, Austria) at a wavelength of 450 nm, OD_{450} (by the intensity of alcohol staining with the extracted dye).

Statistical data analysis. The data were processed by methods of variation statistics using the Statistica 13.3 software package. The statistical significance of the differences was assessed using Student's t-test (reliability criterion). Differences at the p level < 0.05 were considered reliable. The correlation between the studied parameters was assessed using the Pearson correlation coefficient.

RESULTS AND DISCUSSION

In this study we proceed from the hypothesis that *P. aeruginosa* synthesizes alginate to store nutrients necessary to ensure energy metabolism in case of nutritional deficiency, thus resulting in the culture growth. Considering this hypothesis, we should expect a non-linear biofilm formation with a decrease in the amount and concentration of nutrients in the culture medium.

According to the results obtained, the intensity of *P. aeruginosa* biofilm formation varies depending on the concentration of nutrients, the volume of nutrient medium in the well and the growth phase of the culture used for inoculation. When concentration of nutrient media components decreased, differences in biofilm formation were more noticeable and were statistically significant (Table 1).

When a *P. aeruginosa* culture, that had been at a stationary growth phase for 48 hours, was used for inoculation, the biofilm formation depended only on concentration of nutrients in the volume of cultivation (Fig. 1). Thus, when the LB medium was twice diluted, the volume of

Table 1
Growth of *P. aeruginosa* ATCC 9027 biofilms under different cultivation conditions, OD_{450}, M ± SD (*n* = 4)

Growth phase of *P. aeruginosa* ATCC 9027 culture/ nutrient medium	Volume of nutrient medium in wells, ml							
	1.0				0.2			
	Concentration of nutrient media, %							
	50	25	12.5	6	50	25	12.5	6
Stationary phase/ LB	0.16 ± 0.00	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.15 ± 0.02	0.16 ± 0.01***	0.15 ± 0.02***	0.08 ± 0.01***
Logarithmic phase/ Schaedler	0.25± 0.02	0.16 ± 0.02	0.16 ± 0.01	0.13 ± 0.01	0.31 ± 0.05*	0.26 ± 0.07**	0.25 ± 0.03**	0.14 ± 0.02
Logarithmic phase/ LB	0.18 ± 0.04	0.17 ± 0.04	0.16 ± 0.03	0.12 ± 0.03	0.17 ± 0.03	0.16 ± 0.02	0.14 ± 0.02**	0.11 ± 0.01

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

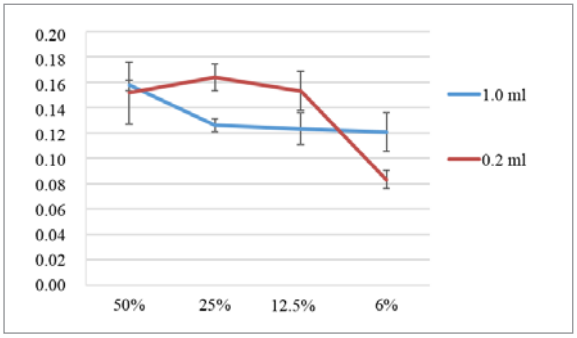

Fig. 1. Growth of P. aeruginosa ATCC 9027 biofilms (inoculation of culture grown up to a stationary phase) in wells containing 0.2 and 1.0 ml of LB medium and different concentrations of nutrients, OD_{450} (M ± SD)

cultivation did not affect the intensity of biofilm growth. After a 4–8-fold dilution of the nutrient medium *P. aeruginosa* formed a biofilm of higher density, when grown in wells with 1.0 ml of broth, than when grown in 0.2 ml of medium ($p = 0.000232$ and 0.000129 respectively). Within this range of nutrient medium concentrations, the intensity of biofilm formation was the same and demonstrated a statistically significant decrease in 0.2 ml cultivation volume in comparison with the culture grown in 1.0 ml of nutrient medium ($p = 0.000825$).

As Figure 2 shows, when *P. aeruginosa* was used for inoculation at a log phase (after a series of passages in this phase of cultivation), the intensity of biofilm formation was also influenced by the concentration of nutrients in the Schaedler medium. It should be noted that 0.2 ml cultivation volume implies a 5-fold difference in the total nutrient content compared to 1.0 ml. Accordingly, all the dependence curves did not intersect, biofilm formation in 0.2 ml cultivation volume was naturally less at all concentrations of nutrients in comparison with cultures grown in 1.0 ml volume.

After a 8-fold dilution of the nutrient medium, there is a sharp decrease in the efficiency of biofilm matrix synthesis, both when cultured in 1.0 ml volume and when grown in 0.2 ml volume. It is confirmed by the fact that the dependence of biofilm formation on the nutrient content is more accurately described by a two-step polynomial function ($R^2 = 0.996–0.979$) than by a linear function ($R^2 = 0.854–0.931$).

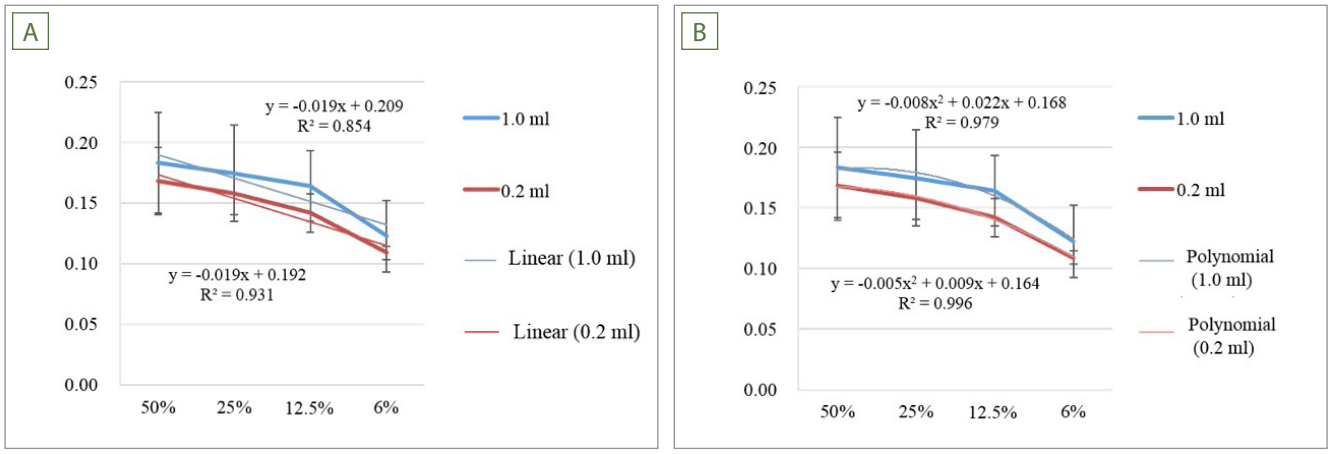

Fig. 2. Growth of P. aeruginosa ATCC 9027 biofilms (inoculation of culture grown up to a log phase) in wells containing 0.2 and 1.0 ml of LB medium and different concentrations of nutrients, OD_{450} (M ± SD): A – linear trend; B – polynomial trend

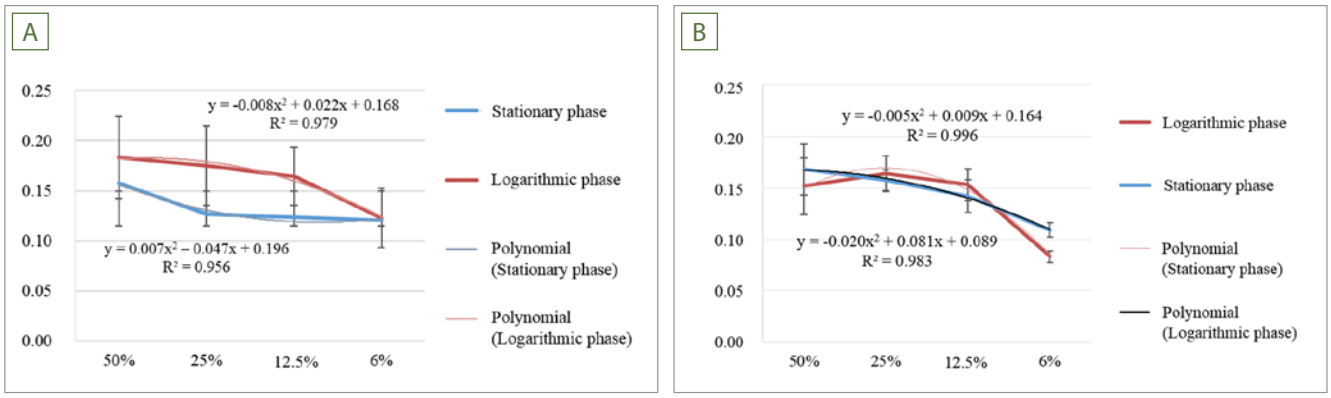

Fig. 3. Growth of P. aeruginosa ATCC 9027 biofilms (inoculation of cultures grown up to log and stationary phases) in wells containing different volumes of LB medium and different concentrations of nutrients, OD_{450} (M ± SD): A – 1.0 ml; B – 0.2 ml

The effect of *P. aeruginosa* growth phase used for cultivation on biofilm formation was most pronounced when cultured in 1.0 ml volume using LB nutrient medium diluted 4 and 8 times ($p = 0.0209$ and 0.0053 respectively). Such dependence was not observed when cultivating these bacteria in 0.2 ml (Fig. 3).

It is natural that a metabolically more active culture at a log phase used the components of the nutrient medium more efficiently. At the same time, the amount of nutrients extracted from 1.0 ml cultivation volume was greater than from 0.2 ml.

The non-linear nature of biofilm formation, in relation to the concentration and total content of nutrients in the culture medium, allows assuming that biofilms are connected with the ability to stockpile and concentrate nutrients. Differences in the intensity of biofilm synthesis associated with the culture growth phase during inoculation, suggest that they are caused not only by genetic variability, but also by the ability to preserve the phenotype due to the epigenetic mechanisms of the quorum sensing in *P. aeruginosa*.

Cultivation of static biofilms in liquid nutrient media resulted in a conclusion that biofilm formation depended on concentration of nutrients, the medium volume and the growth phase of the culture used for cultivation.

As the research demonstrates, a bacterial growth phase, where *P. aeruginosa* culture functions at the moment of inoculation, is a decisive factor in the biofilm formation. When inoculating *P. aeruginosa*, which had been at the stationary growth phase for 48 hours, the biofilm formation showed non-linear dependence on concentration of nutrients and on their total amount in the cultivation volume. Two-fold dilution of the LB nutrient medium did not affect the biofilm growth; however, a 4- to 8-fold decrease in concentration of nutrients in the 1.0 ml cultivation volume stimulated the biofilm formation.

In 1.0 ml cultivation volume, the intensity of biofilm formation was even and did not depend on the degree of the LB medium dilution, and in 0.2 ml cultivation volume it demonstrated a statistically significant decrease, when diluted 4 and 8 times.

The linear dependence of biofilm formation on concentration of nutrients in Schaedler culture medium was more pronounced, when inoculating *P. aeruginosa* at a log phase. The dependence graphs were described by a linear function with a correlation coefficient $R^2 = 0.854–0.931$.

The cultures grown in a smaller cultivation volume (0.2 ml) at different concentrations of nutrients formed biofilms of lower density compared to microorganisms cultivated in 1.0 ml.

Studying the influence of logarithmic and stationary phases of *P. aeruginosa* culture growth on biofilm formation revealed that the biofilms formed more intensively in 1.0 ml cultivation volume. At the same time, a metabolically more active culture inoculated at a log growth phase used the culture medium nutrients more efficiently.

The study of the effect of nutrient concentration and total nutrient content in the cultivation volume on biofilm formation resulted in an assumption that biofilm formation is connected with the function of storing and concentrating nutrient components of the medium. At the same time, it is important to note non-linear nature of the biofilm formation, which confirms the hypothesis that if there is a lack of nutrients promoting its growth, *P. aeruginosa*is able to synthesize alginate which helps to preserve and maintain the concentration of nutrients needed for the population growth. In addition, due to depletion of nutrients alongside with accumulation of metabolic products, the vital activity of all biofilm-forming microorganisms is inhibited, while at low concentrations of nutrients this process naturally decreases.

CONCLUSION

The described study demonstrates that *P. aeruginosa* biofilm formation depends both on the culture growth phase used for inoculation, on the volume of the cultivation well, and on the concentration of nutrients in the cultivation volume.

The research reveals that the culture growth phase, at which microorganisms functioned before being inoculated into liquid nutrient media, was a factor of paramount importance. Determination of optical densities of the studied samples showed, when *P. aeruginosa* was inoculated at a log growth phase, the linear dependence of the biofilm formation on concentration of nutrients in the cultivation volume was more pronounced in comparison with the culture at a stationary growth phase. When *P. aeruginosa* grown up to a stationary phase was used for inoculation,

we observed a uniform growth of biofilms at different concentrations of nutrient components in the medium and cultivation volumes.

Bacterium biofilm formation at a log growth phase in 0.2 ml wells with a decreased concentration of nutrients (50; 25; 12.5 and 6%) in the liquid nutrient Schaedler medium was characterized by the following growth indicators: 0.31 ± 0.05; 0.26 ± 0.07; 0.25 ± 0.03; 0.14 ± 0.02; biofilm growth indicators in 1.0 ml cultivation volume were: 0.25 ± 0.02; 0.16 ± 0.02; 0.16 ± 0.01; 0.13 ± 0.01. When cultivating *P. aeruginosa* in a liquid nutrient LB medium, a pronounced linear dependence of the biofilm formation on the concentration of nutrients was noted. In 0.2 ml wells, the biofilm growth rates were as follows: at a 50% concentration of nutrient media –0.17 ± 0.03; at 25% –0.16 ± 0.02; at 12.5% – 0.14 ± 0.02; at 6% – 0.11 ± 0.01. In 1.0 ml wells, these indicators were: at a 50% medium concentration – 0.18 ± 0.04; at 25% – 0.17 ± 0.04; at 12.5% – 0.16 ± 0.03; at 6% – 0.12 ± 0.03. It was also found that in 1.0 ml cultivation volume the intensity of biofilm formation was uniform, and in 0.2 ml, there was a statistically significant decrease arising from a 4- and 8-fold dilution of the nutrient medium. At the same time, the 4- and 8-fold decrease in the concentration of nutrients in 1.0 ml cultivation volume stimulated the biofilm formation.

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