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THE INFLUENCE OF TEMPERATURE AND PH OF THE ENVIRONMENT ON THE PROCESSES OF BIOFILM FORMATION IN GRAM-NEGATIVE NON-FERMENTING BACTERIA

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Abstract

Introduction. *P. aeruginosa* and *A. baumannii* are important subjects of research into biofilm formation processes under various conditions. The formation of the biofilm phenotype is regulated by quorum-sensing signals under the influence of a complex of environmental factors, including osmotic pressure, temperature, pH, availability of carbohydrate substrates and aeration levels. The interaction between quorum-sensing regulation and environmental conditions is complex and multifactorial. Knowledge of the optimal environmental parameters for bacterial biofilm formation is important for the development of effective control strategies.

Aim. To determine the effect of pH and ambient temperature on the intensity of biofilm formation by clinical strains of *P. aeruginosa* and *A. baumannii*.

Materials and methods. The study utilized 10 clinical strains of each bacterial species, isolated from wound exudate. The effect of ambient temperature (27 °C, 32 °C, 37 °C, 39 °C) and pH values (5.0, 7.0, 8.0) on the intensity of bacterial biofilm formation was assessed.

Results. It was found that in *P. aeruginosa* and *A. baumannii*, biofilm formation occurs more intensively at temperatures lower than human body temperature (27 °C and 32 °C, respectively). An increase in temperature to 39 °C is accompanied by inhibition of biofilm formation processes in both species studied. The optimal conditions for *P. aeruginosa* biofilm formation are neutral pH values of the culture medium. *A. baumannii* intensifies the biofilm formation process in a slightly alkaline medium (pH 8.0).

Conclusions. Temperature and hydrogen ion concentration in the medium play a key role in regulating biofilm formation by *P. aeruginosa* and *A. baumannii*. Knowledge of the parameters influencing the intensity of biofilm formation must be utilized in the development of effective strategies for the treatment and control of nosocomial infections caused by these pathogens.

Keywords: *P. aeruginosa*, *A. baumannii*, biofilms, resistance, temperature effect, pH of the nutrient medium

INTRODUCTION

The study of bacterial biofilm formation is of substantial practical importance, since within biofilms bacteria acquire properties that are absent in their planktonic forms. Biofilms are structured consortia of microorganisms attached to biotic or abiotic surfaces. They consist of functionally heterogeneous cells retained within a matrix formed by extracellular polymeric substances produced by the bacteria. The presence of an extracellular polymeric matrix increases microbial resistance to host immune defences, antimicrobial agents, physicochemical environmental influences, and other factors [1, 2].

The importance of bacterial biofilm formation in medical practice is stipulated by the fact that,

in many bacterial diseases, particularly those with a chronic course, such as cystic fibrosis, periodontitis, rhinosinusitis, osteomyelitis, trophic ulcers, and others, the formation of bacterial biofilms adversely affects treatment effectiveness. The formation of biofilms on the surfaces of medical implants, urinary catheters, and other medical devices is particularly dangerous. Ultimately, the formation of bacterial biofilms on various surfaces within the internal environment of healthcare facilities creates reservoirs of nosocomial infections that are difficult to eliminate by conventional disinfection measures [3, 4].

Gram-negative non-fermenting bacteria, in particular *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, are characterized by high biofilm-forming activity and,

due to their high level of antibiotic resistance, have been included by the World Health Organization in the list of priority pathogens for the development of new antibiotics [5]. These bacterial species have become leading causative agents of wound and burn infections, as well as severe healthcare-associated infections, including ventilator-associated pneumonia with high mortality. Owing to their high adhesive capacity, they readily colonize the hospital environment and, within biofilms protected by the polysaccharide matrix, are able to withstand exogenous stresses caused by antibiotics, disinfectants, moisture deficiency, and similar factors [6, 7, 8].

The transition of planktonic forms of *P. aeruginosa* and *A. baumannii* to biofilm formation is regulated by the quorum-sensing system under the influence of environmental factors, as a response aimed at enhancing microbial population survival. Biofilm formation depends on numerous factors, including nutrient availability, humidity, osmotic pressure, ambient temperature, and others. Under the influence of these factors, the formation of the adhesion apparatus, synthesis of matrix polysaccharides, and functional differentiation of cells are expressed [9, 10]. Knowledge of the optimal environmental parameters for bacterial biofilm formation, as well as factors destructive to biofilms, is important for the development of rational antimicrobial therapy regimens and effective protocols for environmental decontamination.

AIM

The aim of this study was to determine the effect of medium pH and cultivation temperature on the intensity of biofilm formation by clinical strains of *P. aeruginosa* and *A. baumannii*.

MATERIALS AND METHODS

The study was conducted at the research bacteriological laboratory of the Department of Microbiology, National Pirogov Memorial Medical University, Vinnytsya. Clinical strains of *P. aeruginosa* and *A. baumannii* were used in the study, with 10 isolates of each species obtained from patients with burn injuries and mine-blast wounds who were treated at M. I. Pirogov Vinnytsia Regional Clinical Hospital. Microorganisms were identified using standard bacteriological methods, taking into account their morphological, tinctorial, and biochemical characteristics.

The intensity of biofilm formation was studied during cultivation of the bacterial strains in tryptic soy broth (TSB) for 24 hours at temperatures of 27 °C, 32 °C, 37 °C, and 39 °C.

The effect of medium pH on biofilm-forming activity was assessed using an isotonic buffered peptone solution with an initial pH of 7.0. The hydrogen ion concentration in the solution was adjusted by stepwise addition of 1 N NaOH or H₂SO₄ solutions under pH-metric control.

The biofilm-forming capacity of the clinical isolates was determined by the spectrophotometric method (microtiter plate test), which involves biofilm formation in polymer multiwell plates followed by staining with 1% crystal violet solution [11]. Optical density (OD) was measured using a GBG ChroMate 4300 microplate reader (Awareness Technology, Inc., USA) at a wavelength of 630 nm. OD values for each strain and under each set of cultivation conditions were determined in triplicate. Arithmetic mean values (M) and standard errors of the mean (m) were calculated for each dataset.

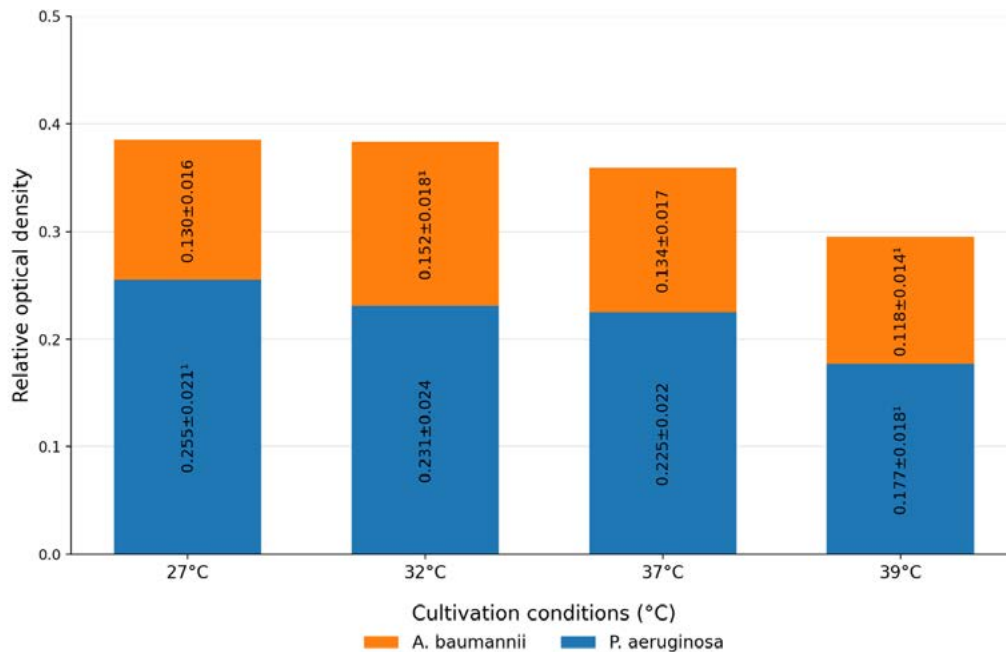
RESULTS

The experimental study demonstrated that the intensity of biofilm formation in clinical strains of *P. aeruginosa* and *A. baumannii* depends significantly on both cultivation temperature and hydrogen ion concentration in the nutrient medium. In both studied species, changes in the physicochemical parameters of the medium were associated with pronounced fluctuations in the optical density values of the formed biofilm, indicating different sensitivities of these microorganisms to temperature and acid-base conditions.

The experiment showed that the peak biofilm-forming activity of non-fermenting gram-negative bacteria occurred at a medium temperature somewhat lower than the physiological temperature of the human body (Fig. 1). Analysis of the effect of temperature revealed that temperatures below physiological body temperature were the most favorable for the development of the biofilm phenotype in both species studied. At the same time, the pattern of temperature response in *P. aeruginosa* and *A. baumannii* displayed certain species-specific features, indicating differences in the adaptive mechanisms of these pathogens to environmental conditions.

The peak biofilm biomass in *P. aeruginosa* (OD = 0.255 ± 0.021) was recorded at 27 °C. Under these conditions, the mass of the formed biofilm was the highest. Moreover, this optical density value differed significantly ($p < 0.05$) from that obtained under cultivation at 37 °C. These findings indicate that lowering the cultivation temperature to 27 °C creates the most favorable conditions for the accumulation of biofilm biomass by clinical strains of *P. aeruginosa*.

With an increase in temperature by 5 °C, a decrease in the intensity of biofilm formation by approximately 10% was observed. At 32 °C, *P. aeruginosa* demonstrated a reduction in biofilm-forming intensity. The optical density value at 32 °C (OD = 0.231 ± 0.024) did not differ significantly from that obtained at 37 °C. Nevertheless, this reflects a tendency toward gradual weakening of biofilm-forming activity as temperature increases. Thus, for *P. aeruginosa*, the temperature range from 32 °C to 37 °C may be regarded as a zone of moderate biofilm-forming activity, whereas its most pronounced manifestation was observed specifically at 27 °C.



Note: 1 - difference is significant ($p < 0.05$) compared with growth of the same culture at 37°C.

Figure 1. Biofilm formation intensity under different cultivation temperature conditions.

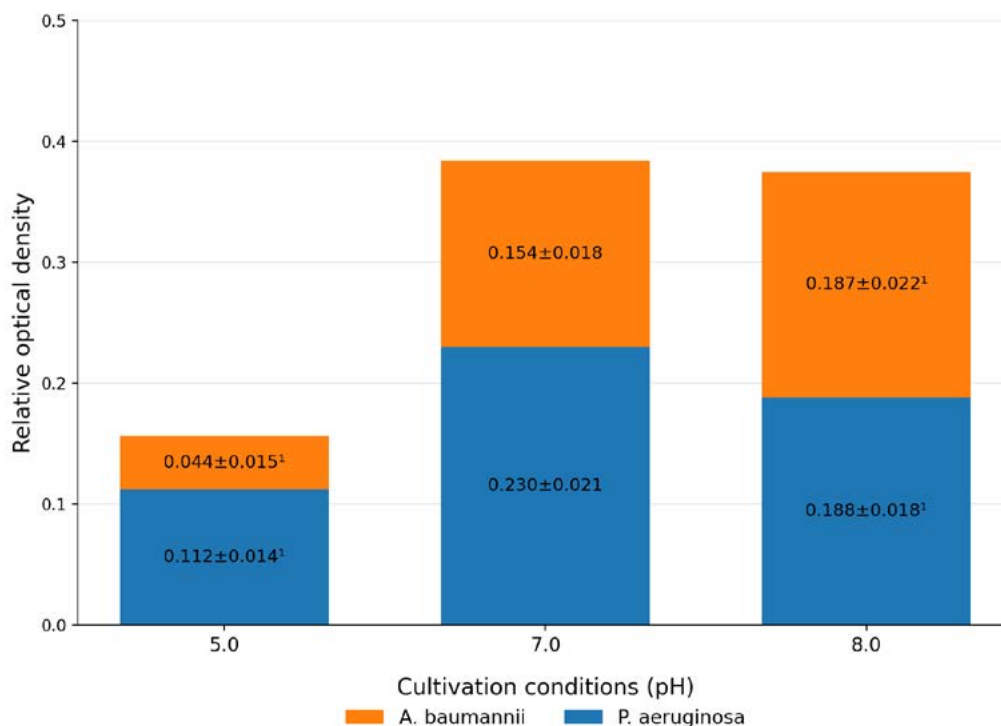
A. baumannii also exhibited maximal biofilm-forming activity at temperatures below 37 °C, although at values higher than those optimal for *P. aeruginosa*. In *A. baumannii*, temperature dependence was likewise characterized by more intense biofilm formation at temperatures below 37 °C; however, unlike *P. aeruginosa*, the maximal activity of this species was recorded at 32 °C. At 27 °C, the optical density of biofilms formed by *A. baumannii* did not differ significantly from that observed at 37 °C, although it was slightly lower. This indicates that 27 °C is not the optimal temperature for biofilm formation in *A. baumannii*, although it does not exert a marked inhibitory effect on this process. The most intensive biofilm formation in this species was observed at 32 °C. Therefore, comparative analysis of the temperature-testing results showed that both bacterial species exhibited maximal biofilm-forming capacity at temperatures below 37 °C; however, the location of the temperature optimum did not coincide. In *P. aeruginosa*, the highest intensity of the process was observed at 27 °C, whereas in *A. baumannii* it was observed at 32 °C. This suggests the existence of species-specific temperature preferences in biofilm formation.

In representatives of both species, a marked decrease in the intensity of biofilm formation was observed when the temperature was increased to 39 °C. At the same time, both studied microorganisms demonstrated a common pattern: cultivation at 39 °C was accompanied by a substantial reduction in biofilm-forming intensity. The decrease in biofilm biomass under these conditions indicates suppression of biofilm formation processes as the temperature approached the upper limit of the studied range. This may be associated with reduced biofilm-

forming activity at elevated temperatures, although accelerated biofilm maturation followed by transition to the dispersal phase cannot be excluded. Regardless of the mechanism, 39 °C was the least favorable condition for biofilm biomass accumulation in both species.

The results of the study on the effect of medium pH on the intensity of biofilm formation demonstrated differences in the response of the two bacterial species to deviations from neutral pH (Fig. 2). The findings also revealed substantial interspecies differences in the nature of the adaptive response of *P. aeruginosa* and *A. baumannii*. Changes in the acid-base status of the cultivation medium were accompanied by multidirectional alterations in biofilm formation intensity, which makes it possible to regard pH as one of the important regulatory factors in the expression of the biofilm phenotype in the non-fermenting gram-negative bacteria under study.

Acidification of the nutrient medium to pH 5.0 led to a decrease in the intensity of biofilm formation in both studied species, although to different extents: in *P. aeruginosa*, the optical density of the formed biofilm decreased twofold, whereas in *A. baumannii* it decreased more than threefold compared with the optical density observed under neutral pH conditions. This indicates marked suppression of biofilm formation under acidic conditions. At the same time, even under such conditions, *P. aeruginosa* retained a certain capacity for biofilm formation, although at a substantially lower level. For *A. baumannii*, the acidic medium proved to be even less favorable. Thus, *Acinetobacter* demonstrated greater sensitivity to acidification of the medium than *P. aeruginosa*. This pattern suggests that acid stress imposes a more pronounced limitation on the biofilm-forming potential of *A. baumannii*.



Note: 1 - difference is significant ($p < 0.05$) compared with growth of the same culture at pH 7.0.

Figure 2. Biofilm formation intensity under different medium pH conditions.

P. aeruginosa exhibited the highest biofilm-forming activity ($OD = 0.230 \pm 0.021$) in a neutral nutrient medium (pH 7.0). A neutral medium proved to be optimal for biofilm formation by *P. aeruginosa*. Specifically, neutral pH provided the most favorable conditions for the accumulation of biofilm biomass in pseudomonads. At the same time, deviations in pH toward either the acidic or alkaline range were accompanied by a decrease in the intensity of this process.

A shift in medium pH toward alkalinity (pH 8.0) was associated with a statistically significant decrease in the intensity of biofilm formation in pseudomonads. This indicates that, for this species, even moderate alkalization of the medium is less favorable for the expression of the biofilm phenotype than neutral conditions. Therefore, for *P. aeruginosa*, a clearly defined optimum at neutral pH can be stated.

Unlike *Pseudomonas*, *A. baumannii* formed biofilms most intensively ($OD = 0.187 \pm 0.022$) in an alkaline medium. In contrast to *P. aeruginosa*, *A. baumannii* demonstrated a different pattern of response to changes in the acid-base status of the medium. The most intensive biofilm formation in this species was observed specifically at pH 8.0, that is, under mildly alkaline conditions. Thus, for *A. baumannii*, alkalization of the medium not only did not limit biofilm formation, but, on the contrary, proved to be the most favorable factor among the tested conditions.

Generalization of the obtained data allows the conclusion that the responses of the two studied species

to pH changes are fundamentally different. Whereas neutral pH values are optimal for *P. aeruginosa*, a mildly alkaline medium is the most favorable for *A. baumannii*. At the same time, an acidic medium suppresses biofilm formation in both species, although more markedly in *A. baumannii*. This highlights species-specific features of adaptation of non-fermenting gram-negative bacteria to changes in the acid-base status of the environment.

Thus, the results of the present study demonstrated that both cultivation temperature and nutrient medium pH substantially modulate the intensity of biofilm formation in clinical strains of *P. aeruginosa* and *A. baumannii*. For *P. aeruginosa*, the most favorable conditions were a temperature of 27 °C and a neutral pH of 7.0, whereas for *A. baumannii* the optimal conditions were a temperature of 32 °C and a mildly alkaline medium with a pH of 8.0. An increase in temperature to 39 °C, as well as acidification of the medium to pH 5.0, was accompanied by inhibition of biofilm formation in both studied species.

DISCUSSION

Due to their high adaptive capacity, gram-negative non-fermenting bacteria have become ubiquitous microorganisms capable of surviving under extreme conditions and infecting numerous living hosts. Transition to the biofilm form represents not only one of the mechanisms of adaptation, but also a pathogenicity factor. Observations have been reported regarding an

inverse correlation between biofilm-forming activity and the presence of antibiotic resistance mechanisms in *A. baumannii*, which illustrates the complexity of compensatory survival mechanisms [12, 13].

Given the diversity of ecological niches inhabited by *P. aeruginosa* and *A. baumannii*, temperature is one of the key factors determining whether these bacteria exist in the form of free-living planktonic cells or as biofilms. Under different temperature conditions, the biofilm biomass, exopolysaccharide content, as well as its structure and morphology change, thereby affecting functional properties [14, 15]. The results of our study are consistent with previous findings indicating that peak biofilm formation in *Acinetobacter* and *Pseudomonas* occurs within the temperature range of 25 °C to 30 °C. At temperatures above approximately 32 °C, these bacterial species activate the transition to the planktonic form, as well as reproductive and enzymatic processes [16]. These biological features of non-fermenting gram-negative bacteria should be taken into account, first of all, when developing protocols for decontamination of healthcare facility premises. Washing surfaces with water at room temperature may promote the formation of pathogen biofilms, within which the microorganisms become less susceptible to disinfectants.

Studies investigating the effect of medium pH on biofilm formation are limited, and their results are often contradictory. Most bacterial pathogens of humans do not survive in environments with a pH below 4.5. According to some studies, non-fermenting bacteria activate biofilm formation in neutral or mildly acidic environments [17, 18, 19]. In our study, biofilm formation was assessed in an acidic medium (pH 5.0), and it was established that under such pH conditions biofilm formation is inhibited. The obtained results confirm the conclusions of previous studies indicating that *P. aeruginosa* exhibits the highest biofilm-forming intensity at neutral pH. Of particular interest is the observed enhancement of biofilm formation by *A. baumannii* in an alkaline medium (pH 8.0). This finding may explain why burn wounds, the environment of which is predominantly alkaline, become persistently colonized by *A. baumannii* at early stages, whereas infection caused by *P. aeruginosa* usually develops later, when the pH of wound exudate approaches neutrality [20].

CONCLUSIONS

Temperature conditions and hydrogen ion concentration in the medium play a key role in the regulation of biofilm formation by *P. aeruginosa* and *A. baumannii*. The results of the study showed that the intensity of biofilm formation in clinical strains of both species depends substantially on the physicochemical parameters of the cultivation environment, while the response of the studied microorganisms to changes in temperature and pH is species-specific.

It was established that the most favorable conditions for biofilm formation by *P. aeruginosa* are a temperature of 27 °C and a neutral pH of 7.0, whereas for *A. baumannii* the optimal conditions were a temperature of 32 °C and a mildly alkaline medium with a pH of 8.0. This indicates differences in the adaptive mechanisms of these pathogens and in the way they express the biofilm phenotype depending on environmental conditions.

An increase in temperature to 39 °C was accompanied by inhibition of biofilm formation in both studied species, while acidification of the medium to pH 5.0 also reduced the intensity of this process, with a more pronounced effect in *A. baumannii*. Therefore, both temperature and the acid-base status of the medium can substantially limit the ability of clinical isolates to form biofilms.

Knowledge of the parameters influencing biofilm formation intensity should be used in the development of effective strategies for the treatment and control of nosocomial infections caused by these pathogens. The obtained data are of practical importance for improving approaches to the prevention of persistence of *P. aeruginosa* and *A. baumannii* in the clinical environment, as well as for substantiating measures aimed at reducing the risk of formation of stable biofilm-associated communities.

Prospects for further research. Further studies are warranted to investigate in greater depth the interaction of temperature and acid-base status of the medium with other factors influencing biofilm formation by *P. aeruginosa* and *A. baumannii*, including cultivation duration, nutrient availability, aeration, and susceptibility to antimicrobial agents and disinfectants. Such studies will allow a more precise determination of the adaptive mechanisms of these pathogens and support the development of more effective approaches to the prevention and control of nosocomial infections.

COMPLIANCE WITH ETHICAL REQUIREMENTS

The study was conducted in accordance with generally accepted ethical principles of biomedical research, biosafety requirements, and good laboratory practice. Clinical microbial isolates obtained during routine microbiological diagnostics were used in the study.

No personally identifiable patient data were used in the study, which ensured compliance with the principles of confidentiality and anonymity.

During the preparation of this work, artificial intelligence technologies were not used for text writing, data processing and analysis, or image generation. The entire content of the article was prepared by the authors, who bear full responsibility for its scientific accuracy, reliability, and correctness.

FUNDING AND CONFLICT OF INTEREST

The study was conducted without external funding. The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

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Резюме

ВПЛИВ ТЕМПЕРАТУРИ ТА PH СЕРЕДОВИЩА НА ПРОЦЕСИ БІОПЛІВКОУТВОРЕННЯ У ГРАМНЕГАТИВНИХ НЕФЕРМЕНТУЮЧИХ БАКТЕРІЙ *P. AERUGINOSA* ТА *A. BAUMANNII*

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Вступ. *P. aeruginosa* та *A. baumannii* є важливими об'єктами дослідження процесів біоплівкоутворення за різних умов. Формування біоплівкового фенотипу регулюється кворум-сенсинговими сигналами під впливом комплексу чинників навколишнього середовища, зокрема осмотичним тиском, температурою, значенням рН, доступністю вуглеводних субстратів і рівнем аерації. Взаємодія між кворум-сенсинговою регуляцією та умовами навколишнього середовища має складний і багатофакторний характер. Знання оптимальних параметрів середовища для утворення бактеріями біоплівок важливе для розробки ефективних режимів боротьби з ними.

Мета. Визначення впливу рН і температури оточуючого середовища на інтенсивність утворення біоплівок клінічними штамми *P. aeruginosa* та *A. baumannii*.

Матеріали та методи. У дослідженні використано по 10 клінічних штамів обох видів бактерій, виділених з вмісту ран. Оцінювали вплив температури навколишнього середовища (27 °C, 32 °C, 37 °C, 39 °C) і значень рН середовища (5,0, 7,0, 8,0) на інтенсивність утворення бактеріальних біоплівок.

Результати. Встановлено, що у *P. aeruginosa* та *A. baumannii* біоплівкоутворення відбувається інтенсивніше за температур, нижчих за температуру тіла людини (27 °C і 32 °C відповідно). Підвищення температури до 39 °C супроводжується пригніченням процесів біоплівкоутворення в обох досліджуваних видів. Оптимальними умовами формування біоплівок *P. aeruginosa* є нейтральні значення рН поживного середовища. *A. baumannii* інтенсифікують процес біоплівкоутворення у слаболужному середовищі (8,0).

Висновки. Температурний режим і концентрація водневих іонів у середовищі відіграють ключову роль у регуляції біоплівкоутворення *P. aeruginosa* та *A. baumannii*. Знання параметрів впливу на інтенсивність біоплівкоутворення необхідно використовувати в процесі розробки ефективних стратегій лікування та контролю нозокоміальних інфекцій, обумовлених цими збудниками.

Ключові слова: *P. aeruginosa*, *A. baumannii*, біоплівки, резистентність, вплив температури, рН середовища

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