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SCREENING OF THE ABILITY OF MICROORGANISMS TO BIOFILM FORMATION

Summary

Biofilms are an important element of microbial communities and perform various functions determined by their composition, structure and environmental conditions in which they are formed. Biofilms can form on various surfaces, such as soil, glass, metal, polymeric materials, and biostructures of living organisms. This process takes place with the participation of many species of microorganisms, in particular, bacteria and fungi, which, in this manner, acquire additional mechanisms for survival, in particular, in the presence of antibiotics. To test the biofilm-forming ability of bacteria (Escherichia coli, Bacillus subtilis, Lactobacillus brevis, Rhodococcus luteus, Staphylococcus albus, Azotobacter chroococcum, Pseudomonas fluorescens, Micrococcus luteus) and yeasts (Candida pseudotropicalis, C. curvata, C. kefyr, C. parapsilosis, C. tenuis, Cryptococcus lactativorus, Cryptococcus laurentii), which were affected by plant extracts, three methods were used. The ability of these microorganisms to form biofilms was not detected.

Key words: microorganisms, biofilms, ability to form biofilms.

Introduction. The persistence of bacteria in the environment is often ensured precisely by the ability to form biofilms. These structures make it possible to adapt, attach and survive in conditions of constant change. Biofilms are a barrier to protect microorganisms from adverse conditions such as degradation, desiccation, UV radiation, and exposure to chemical compounds [1,2]. They can help retain and stabilize microorganisms in appropriate environments, providing optimal conditions for their existence and functioning and adhesion to various surfaces [3,4,5], which can be important for the formation of biological communities and biogeochemical cycles. These structures can interact with the environment, including the exchange of substances with other microorganisms, the formation of covalent or non-specific bonds with chemical compounds [6,7,8].

It is known that Bacillus can survive in extreme conditions by forming spores. Spore formation occurs within biofilms, which, for example, B. subtilis form as three-dimensional architectural structures. It is B. subtilis that is often used as a model for studying the biofilms formation [6,9]. Different species of microorganisms’ form biofilms of different chemical compositions [2,10,11] which are investigated using different methods [12,13].

Escherichia coli and Staphylococcus epidermidis, as commensal bacteria with high reproductive and pathogenic potential [14,15], are often resistant to biotic and abiotic factors [16] due to biofilm formation [17,18].

Micrococcus luteus are catalase-, oxidase-, and gram-positive cocci that grow on media in the form of yellow or golden colonies, which is associated with the production of carotenoid pigments. They are usually part of the normobiota capable of causing human disease under certain conditions [19]. M. luteus known for its adaptation and survive in extreme conditions and the ability to withstand desiccation, which makes the resistant in a variety of environments and facilitate the preservation and spread in the environment. Alone or together with Staphylococcus and Bacillus,
Micrococcus are major contaminants of clean premises of pharmaceutical factories [20,21].

In addition to their natural environment, many species of bacteria and fungi, such as Pseudomonas, Staphylococcus or Candida, have the ability to form stable biofilms in the living organism, which is considered one of the main causes of most infectious diseases [22,23,24,25,26,27]. They colonize various surfaces, including medical implants [28,29,30,31], threatening patients who have undergone invasive medical interventions.

Microorganisms have various virulence factors, such as adhesins and membrane proteins, that enable bacterial attachment, colonization, and invasion of host tissues. In addition, microbial toxins cause damage to host tissues, and components of the bacterial cell wall, such as capsular polysaccharide, ensure the resilience to counteracts of the host's immune system. Biofilm formation and the ability to sporulate are additional virulence factors that contribute not only to pathogenesis, but also to pathogen resistance to therapeutic agents [32,33,34,35,36].

Biofilm-forming bacteria have various manners of protecting themselves against antibacterial drugs [24,29,37], and some survive through antibiotic tolerance [2,38,39,40].

Alternatives to antibiotics are sometimes compounds or their mixture isolated from plants, such as phenols and flavonoids, in particular luteolin [41], which in studies have demonstrated antimicrobial and antibiotic activity against Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus, and Escherichia coli [42,43]. The antibacterial mechanism of luteolin against S. aureus includes inhibition of nucleic acid and protein synthesis, damage to the bacterial cell membrane, and induction of the cell morphological change [44].

We found that plants extracts can inhibit the growth of bacteria and yeasts [45,46,47], but the biofilm formation of the studied strains is not known. Therefore, the aim of the work was to test the biofilm-forming ability of 17 strains of the microorganisms of the culture collection of the Department of Microbiology Ivan Franko National University of Lviv.

Research methods. Bacteria (Escherichia coli, Bacillus subtilis, Brevisibacillus brevis, Rhodococcus luteus, Staphylococcus albus, Azotobacterchroococcum, Pseudomonas fluorescens, Micrococcus luteus) and yeasts (Candida pseudotropicalis, C. curvata, C. kefyr, C. tenuis, C. parapsilosis, Cryptococcus lactativorus, Cryptococcus laurentii) were used to study the ability to form biofilms. Three methods were used, which are based on staining the biofilm structures with gentian violet and measuring the absorption optical density.

The first method used was the microplatemethod described by Stepanović et al. (2000) [48]. Suspensions were prepared from one-day cultures for bacteria and two-day cultures for fungi grown on Luria-Bertrani (LB) medium. The suspensions were standardized according to the McFarland turbidity standard of 0.5 (1.5x10^8 cells/ml). The sterile 96-well plate was used, the wells of which were instilled with 0.2 ml of LB medium. Then 0.2 ml of prepared suspensions were added to each well (6 wells per culture). LB-medium was used as control. After 24 hours of incubation in a thermostat at a temperature of 28 ± 1°C, the wells were washed three times with 1M phosphate buffer (pH 7.2) and dried. 0.1 ml of 0.1% gentian violet was added, kept for 25–30 min and washed three times with sterile distilled water. Then 0.2 ml of 96% ethanol was added and measured at 578 nm on a Thermo Scientific Helios™ Epsilon spectrophotometer. After 24 hours of incubation in a thermostat at a temperature of 28 ± 1°C, the wells were washed three times with 1M phosphate buffer (pH 7.2) and dried. 0.1 ml of 0.1% gentian violet was added, kept for 25–30 min and washed three times with sterile distilled water. Then 0.2 ml of 96% ethanol was added and measured at 578 nm on a Thermo Scientific Helios™ Epsilon spectrophotometer.

In the second method, sterile Eppendorf tubes were used as described by Haney et al. (2018) [13]. Sterile culture medium (0.9 ml) was aseptically poured into the tubes and 0.1
ml of bacterial suspension was added. Test tubes were incubated in six replicates for 2 days at a temperature of 28±1°C. Then the contents of the test tubes were poured out, sterile physiological solution was added to the test tubes and washed three times to remove non-adherent cells. The test tubes were dried and biofilms were fixed with 96% ethanol for 20–30 min. After that, 1 ml of 0.1% crystal violet was added, keeping for 30 minutes. The solution was drained, washed and, after drying, filled with 96% ethanol. The optical density of the obtained crystal violet solutions was measured at 578 nm against the solution from the test tube containing only the medium.

In the third method, the ability to form biofilms after incubation of microorganisms in sterile glass Petri dishes with a diameter of 5 cm was investigated (similarly, as described in [49]. 1 ml of suspension was poured into dish (6 dishes for each culture) and left in the thermostat for 3 hours. After 3 hours, 1 ml of liquid Luria-Bertrani medium was added and left in a thermostat at a temperature of 28 ± 1°C for 2 and 10 days. After incubation, they were stained in the same manner as described above.

The results of biofilm formation were classified as: non-biofilm-forming (if the optical density was less than 0.5), with low biofilm formation (if the optical density was less than 2) and moderate biofilm formation (if the optical density was more than 2), similarly to what was proposed by [48].

**Results and their discussion.** After growing test cultures of gram-positive and gram-negative bacteria and yeasts in 96-well plates (Fig. 1), the optical density was measured. Similarly, the ability to form biofilms after incubation of microorganisms in test tubes and Petri dishes was investigated (Fig. 2). The obtained results of optical density values are in the table. 1.

![Fig. 1. View of a 96-well microplate (1 – before incubation; 2 – during staining; 3 – after measurement)](image-url)
Fig. 2. Appearance of Petri dishes (1 – before incubation; 2 – during staining)

### Table 1

<table>
<thead>
<tr>
<th>Strain of microorganisms</th>
<th>Tablet (after 2 days)</th>
<th>Testtubes (after 2 days)</th>
<th>Petridishes (after 10 days)</th>
<th>Petridishes (after 10 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>0.11±0.02</td>
<td>0.13±0.03</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0.13±0.04</td>
<td>0.09±0.04</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Brevibacillus brevis</em></td>
<td>0.14±0.05</td>
<td>0.13±0.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Rhodococcus luteus</em></td>
<td>0.20±0.08</td>
<td>0.09±0.03</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus albus</em></td>
<td>0.12±0.03</td>
<td>0.18±0.03</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Azotobacter chroococcum</em></td>
<td>0.28±0.12</td>
<td>0.18±0.09</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>0.16±0.03</td>
<td>0.12±0.06</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>0.15±0.09</td>
<td>0.13±0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. pseudotropicalis</em></td>
<td>0.16±0.02</td>
<td>0.13±0.02</td>
<td>0.09±0.02</td>
<td>0.11±0.03</td>
</tr>
<tr>
<td><em>C. curvata</em></td>
<td>1.82±0.75</td>
<td>0.89±0.30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>0.11±0.01</td>
<td>0.09±0.14</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td><em>Cryptococcus lactativor</em></td>
<td>1.40±0.14</td>
<td>0.38±0.11</td>
<td>0.15±0.03</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>0.15±0.01</td>
<td>0.13±0.03</td>
<td>0.1±0.02</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td><em>Cryptococcus laurentii</em></td>
<td>0.92±0.31</td>
<td>0.42±0.03</td>
<td>0.16±0.04</td>
<td>0.24±0.04</td>
</tr>
<tr>
<td><em>C. tenuis</em></td>
<td>2.48±0.31</td>
<td>0.48±0.03</td>
<td>0.09±0.01</td>
<td>0.13±0.04</td>
</tr>
<tr>
<td>Control (LB medium)</td>
<td>0.19±0.11</td>
<td>0.07±0.04</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: - – not investigated
As can be seen from the table 1, only two of yeast species (*Candida curvata* and *Cryptococcus lactativorus*) have weak and one (*Candida tenuis*) moderate ability to form biofilms. This was discovered using the tablet method. As for other types of microorganisms studied, no biofilm formation ability was found in them by any method. As for the other species of microorganisms studied, no biofilm for mation ability was detected by any applied method.

**Discussion.** The plate method is one of the most widely used methods for detecting the biofilm-forming ability of the most microorganisms [50,51], as well as others based on the staining of formed biofilms [29,52]. Crystal violet staining itself has its caveats, as this dye is toxic and can cause carcinogenic effects [53,54]. In such studies, it is important to follow certain standard procedures, such as standardization of the starting suspension, although not everything can be standardized, as noted by other researchers [13,50]. However, since many of the restraints of the studied species of microorganisms are capable of biofilm formation [6,9], obviously, the procedure for detecting this ability requires changing the cultivation conditions or nutrientsubstrates that would take into account the specifics of each culture. Having created such conditions, it will be possible to judge the probability of biofilm formation by a certain specific microorganism only in certain conditions [12,13,18], as well as about the fact that it has lost this ability [55].

**Conclusion.** Using the microplate method, it was found that three of yeast species(*Candidacurvata*, *Cryptococcuslactativorus*, *Candidatenuis*) have a moderate ability to form biofilms. This ability has not been confirmed by "test Eppendorf tube" and "glass Petri dish" methods.

**REFERENCES**

survival/
commensalism-to-parasitism-the-dangers-of-underestimating-cleanroom-bacterial-contaminants/


СКРИНИНГ ЗДАТНОСТІ МІКРООРГАНІЗМІВ ФОРМУВАТИ БІОПЛІВКУ

Резюме

Вступ. Біоплівки є важливим елементом мікробних спільнот та виконують різноманітні функції, що визначено їхнім складом, структурою та умовами середовища, в якому вони формуються. Біоплівки можуть утворюватися на різних поверхнях, таких як ґрунт, скло, метал, полімерні матеріали та біоструктури живих організмів. Цей процес відбувається за участю багатьох видів мікроорганізмів, зокрема бактерій та грибів, які, у такий спосіб набувають додаткових механізмів для виживання, зокрема й за наявності антибіотиків.

Нами встановлено, що екстракти з рослин здатні пригнічувати ріст бактерій та дріжджів, однак про біоплівкоутворення досліджених штамів не відомо.

Метою роботи було перевірити здатність до біоплівкоутворення 17 штамів мікроорганізмів з колекції культур кафедри мікробіології ЛНУ ім. Івана Франка.

Методи дослідження. Для перевірки здатності до біоплівкоутворення бактерій (Escherichiacoli, Bacillussubtilis, Lactobacillusbrevis, Rhodococcuslutetus, Staphylococcusalbus, Azotobacterchroococcum, Pseudomonasfluorescens, Micrococcusluteus) та дріжджів (Candidafermentum, C. curvata, C. kefyr, C. parapsilosis, C. tenuis, Cryptococcuslactativorus, Cryptococcuslaurentii), на які впливали екстракти з рослин, використано три методи, які базуються на зафарбовуванні структур біоплівки генціановим фіолетовим і вимірюванні оптичної густини поглинання.

Результати й обговорення. Після вирощування тест-культур грампозитивних та грамнегативних бактерій та дріжджів в 96-лункових планшетах, пробірках і чашках Петрі, зафарбовували утворені біоплівки й було виміряно оптичну густину етанольного розчину десорбованого барвника. Згідно отриманих результатів оптична густина більшості досліджених культур була до 0,5. Це означало, що біоплівок немає. Два види дріжджів (Candida curvata, Cryptococcuslactativorus) мали слабку здатність до біоплівкоутворення з оптичною густинною до 2 одиниць і один (Candidatenuis) – помірну (оптична густина більше 2). Це було виявлено за використання планшетного методу, який є одним з найуживаніших для виявлення здатності до біоплівкоутворення більшості мікроорганізмів. Під час таких досліджень важливо дотримуватися певних стандартних процедур, як от стандартизація стартової суспензії, хоча все стандартизувати неможливо, про що зазначено іншими дослідниками. Очевидно, процедура виявлення здатності до біоплівкоутворення потребує зміни умов культивування чи субстратів живлення, які враховували б особливості кожної культури.

Висновок. За використання мікропланшетного методу було виявлено, що три види дріжджів (Candidacurvata, Cryptococcuslactativorus, Candidatenuis) мають помірну здатність до біоплівкоутворення. Цю здатність не підтверджено «пробірковим» і «чашковим» методами.

Ключові слова: мікроорганізми, біоплівки, здатність до біоплівкоутворення.