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# Effectiveness of nutrient media for the recovery of lyophilisates of fastidious microorganisms: evidence from *Streptococcus spp.*

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**Abstract**. To restore the biological and morphological properties of fastidious bacteria during lyophilization, one of which is a type of streptococcus – bacteria of the genus *Streptococcus*, it is necessary to use expensive specialized nutrient media that are limited in availability for laboratories. In this regard, the purpose of this study was to find the most effective methods of preservation and recovery of fastidious microorganisms using the example of *Streptococcus spp.* The study was performed by a bacteriological method. The isolates of *Streptococcus spp.*, which had the property of alpha- or beta-haemolysis, were selected for the study. The microorganisms were collected as a result of bacteriological research of pathological and biological materials from 20 animals (10 dogs and 10 cats). Microorganisms were determined and counted using Vitek 2 compact, easySpiral, and Scan 500 systems. As a result, the effectiveness of using various combinations of nutrient media for cryopreservation, freeze-drying, and further revitalization of cultures was proven. It was found that the most effective medium for lyophilization is meat-peptone broth with the addition of 5% bovine blood serum, diluted 1:1 with Faibich's medium, and for recovery after lyophilization – meat-peptone broth with the addition of 5% blood serum cattle and 5% glucose. With this combination, the concentration of viable cells corresponded to the limits of $1.7\times10^6$-$2.4\times10^6$ CFU/cm$^3$. The use of other combinations of nutrient media for the revitalization of *Streptococcus* bacteria showed lower efficiency, which corresponded to the concentration of viable cells within $1.2\times10^5$-$2.1\times10^6$ CFU/cm$^3$. The obtained results increase the efficiency of the method of lyophilization of demanding cultures due to combination of non-selective nutrient media and components available in laboratory practice

**Keywords**: bacteria, cryopreservation, lyophilization, culture medium, *Streptococcus*

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## Introduction

Bacteria of the genus *Streptococcus spp*. can cause various diseases, ranging from mild skin infections to necrotizing fasciitis, which can threaten the health of not only animals, but also humans (Belstrom *et al*., 2021). This genus consists of both commensal species that colonize the skin and nose, as well as pathogenic and opportunistic strains that can be isolated for various diseases: upper respiratory tract, pneumonia, meningitis, sepsis, otitis, ophthalmitis, hepatitis, endometritis, and aerosacculitis (Weiser *et al*., 2018; Kim *et al*., 2018; Chang *et al*., 2022).

Some types of streptococci are one of the main colonizers and normal flora of the oral cavity – *Streptococcus mitis* and *S. oralis*, which protect it from inflammatory processes by producing hydrogen peroxide, neutralizing acids, and secreting antimicrobial compounds, competing with opportunistic and pathogenic microorganisms. The competitive properties of these bacteria open the possibility for the use of cultures such as *S. mitis* and *S. oralis* in the bacteriotherapy of diseases in combination with antibacterial preparations (Thurnheer & Belibasakis, 2018; Kim *et al*., 2018; Park *et al*., 2020).

Streptococci are less common in the environment than staphylococci (Belstrom *et al*., 2021). They are not resistant to the influence of physical environmental factors, die quickly, and are demanding to the cultivation conditions. Therewith, if cultivation and storage conditions are not observed, bacteria lose their virulence, which reduces their scientific value for researchers and practical use (Nasran *et al*., 2020).

Success in the commercialization of strains of *Streptococcus spp*. as potential valuable agents for several types of research largely depends on their preservation technologies. Appropriate methods can maintain high cell viability not only during the conservation process, but, most importantly, during the long stay of these strains in collections (Halim *et al*., 2017). Because these bacteria have vulnerable characteristics, freeze-drying under vacuum is often considered one of the best methods for preserving cells (Bircher *et al*., 2018; Bodzen *et al*., 2020).

Freeze drying, or lyophilization, is a dehydration process that stabilizes bacteria to maintain their long-term viability until use. This process is often used for production or valuable collection strains of bacteria (Bodzen *et al*., 2021) and is the most efficient way of long-term storage of microbial strains in collections. The quality of restoration of biological and morphological properties of lyophilized demanding bacteria directly depends on the culture media (Boey *et al*., 2022). The technological stress that occurs during the freeze-drying process can cause cell damage and, in some cases, their death (Bodzen *et al*., 2021). Dehydration of cells caused by osmotic stress due to the addition of protective solutes and freezing itself is a source of mechanical and structural stress on cells and cellular components. During severe dehydration, the plasma membrane can be permeabilized, which is often the cause of cell death. Proceeding from this, the choice of an effective protective environment to reduce the effects of the above factors is a crucial step for effective lyophilization. For this purpose, colloidal substances are used, which reduce the temperature point at which water crystallization occurs. This, in turn, extends the time for cooling the cells and creates conditions for minimizing the negative effect of ice crystals on these microbial cells. Another major step in sublimation drying is rehydration, which precedes the use of bacteria and affects both their survival and functionality (Oslan *et al*., 2017; Bodzen *et al*., 2021).

In the study of Ukrainian scientists Ushkalov *et al*. (2021), who conducted studies on the lyophilization of some pathogenic bacteria, also used the method of different combinations of nutrient media to revitalize cultures, namely a gram-positive coccal culture of *S. aureus*. Therewith, the highest productivity in reconstituted crops was observed upon the use of heart-brain broth.

Haindl *et al*. (2022) investigated the effect of *in vitro* freeze-drying on beneficial faecal microbiota for potential use in the treatment of gastrointestinal diseases. They found that freeze-drying affects cell number, saturation, diversity, and microbial composition, but these effects can be reversible and disappear upon re-cultivation.

Nicco *et al*. (2020) investigated the effect of cryopreservation on the survival of gastric microflora using various temperature conditions from -20°C to -196°C. High survival of microorganisms was noted in the temperature range from -70°C to -120°C.

Meneghel *et al*. (2017) described the effect of several types of sugars on cell membrane stability during freezing of cell cultures. The use of complex sugars resulted in greater destruction of microbial walls than the use of monosaccharides. Similar experimental study with comparable results was performed by Vekeman *et al*. (2013), which also involved the use of distinct types of sugars for cryopreservation of nitrite-oxidizing bacteria.

The purpose of this study was to find the most effective and practical methods for long-term storage and subsequent recovery of alpha- and beta-haemolytic streptococci isolated from dogs and cats.

## Materials and Methods

The study was conducted in 2020-2021 in the Ukrainian Laboratory of Quality and Safety of Products of the agro-industrial complex of the National University of Life and Environmental Sciences of Ukraine and the bacteriological laboratory of the Kyiv Regional Clinical Hospital.

The isolates of *Streptococcus spp*., which had the property of alpha- or beta-haemolysis, were selected for the study. The microorganisms were collected as a result of bacteriological research of pathological and biological materials from 20 animal patients (10 dogs and 10 cats) of the "Multivet" Veterinary Clinic (Kyiv region).

Isolates of *Streptococcus spp*. was collected as follows: using a sterile cotton swab pre-moistened with a 0.2% TWEEN 20 solution (Merck, Germany). Swabs with the selected material were immersed in a test tube with Amies transport medium (BioTesMed, Ukraine) and transported to the bacteriological laboratory. The tampon material was seeded on selective and differential diagnostic nutrient media: 5% blood agar (GRASO, Poland) and meat-peptone broth (HiMedia, India) with the addition of 5% glucose. The inoculated cups were incubated in a thermostat at 37°C for 24 hours. For further research, colonies were selected, which, according to the results of bacterioscopy, were formed by gram-positive coccal microflora. The final identification of microorganisms was carried out using a bacteriological analyser Vitek 2 compact (bioMérieux, France).

Identified cultures of *Streptococcus spp*. underwent the process of cryopreservation and subsequent sublimation (lyophilization) as follows: as a cryoprotectant, Faibich's protective medium was used (gelatine – 1%; sucrose – 10%, distilled water – 89%) in a ratio of 1:1 with broth containing 24-hour cultures of *Streptococcus spp*. Therewith, meat-peptone broth (HiMedia, India) was used in two variations: pure meat-peptone broth and meat-peptone broth with the addition of 5% bovine blood serum to 95% of the broth volume. Each test culture was packed with 1.0 $cm^3$ in bottles with a volume of 5 $cm^3$. Before starting lyophilization, three vials were selected from each experimental batch to establish the typicality, uniformity, and concentration of bacterial mass in the suspension (CFU/$cm^3$). The experimental cultures were cryopreserved in a Forma 700 Series freezer (Thermo Scientific, USA) at -70°C. An LP–3 machine (TelStar, Spain) was used to ensure the lyophilization process. Lyophilization was performed at a deep vacuum of 0.17 mbar and a condenser temperature at 45-50°C.

After 6 months, pre-lyophilized cultures were revitalized using different combinations of nutrient media. For this, the following combinations of nutrient media were tested: pure meat-peptone broth; meat-peptone broth with the addition of up to 95% of the broth volume of 5% bovine blood serum; meat-peptone broth with the addition of 5% glucose to 95% of the broth volume; and meat-peptone broth with the addition of 5% bovine serum and 5% glucose to 90% of the broth volume. After 24 hours, the broth cultures were tested for typicality, uniformity, and concentration of viable microbial cells (CFU/$cm^3$) by seeding on blood agar using the easySpiral system (Interscience, France) and counting on Scan 500 (Interscience, France).

Statistical analysis was performed in a Microsoft Excel 2016 spreadsheet. The Online Epitools Epidemiological Calculator was used to estimate the 95% confidence interval (CI) (Epitools Epidemiological Calculators..., 2018). $P<0.05$ was considered a statistically significant result.

## Results and Discussion

During the study of pathological and biological materials from 20 patient animals (10 dogs and 10 cats), 5 isolates of *Streptococcus spp*. were isolated, which had alpha- (Fig. 1) or beta-haemolysis (Fig. 2) and were used in further research (Table 1).

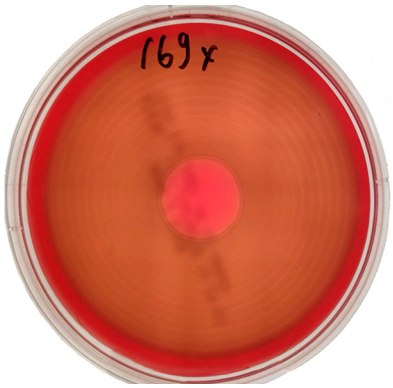


**Figure 1.** Alpha-haemolysis of *S.equinius* isolate on blood agar

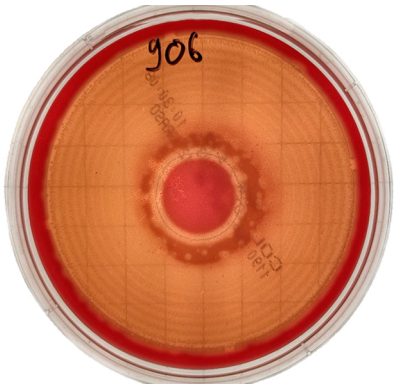


**Figure 2.** Beta-haemolysis of *S.agalactiae* B isolate on blood agar

**Table 1**. Place of isolation, type of haemolysis, and concentration of viable cells of isolates of *Streptococcus spp*. under study

| Indicator | *S. agalactiae* A | *S. agalactiae* B | *S. anginosus* | *S. equinius* | *S. pseudoporcinus* |
|---|---|---|---|---|---|
| Place of isolation | Wound (dog) | Wound (cat) | Mouth (dog) | Oral cavity (cat) | Nasal cavity (dog) |
| Type of haemolysis | *β* | *β* | *α* | *α* | *α* |
| Concentration of viable cells, CFU/$cm^3$ | $1.9 \times 10^8$ | $4.9 \times 10^8$ | $1.6 \times 10^9$ | $1.7 \times 10^9$ | $1.8 \times 10^9$ |

Therewith, the isolates that were taken from the pathological material (from the wound) had a positive reaction to alpha-glucosidase, Ala-Phe-Pro arylamidase, phosphatase and leucine arylamidase according to the results of biochemical analysis on the Vitek 2 compact system and were identified as *S. agalactiae* A, and *S. agalactiae* B. An isolate taken from the pharynx of a healthy animal was positive for D-amygdalin, arginine dihydrolase 1, Ala-Phe-Pro arylamidase, phosphatase and leucine arylamidase and was identified by the system as *S. anginosus*. An isolate taken from the oral cavity of a healthy animal showed a positive reaction to D-amygdalin, cyclodextrin, leucinarylamidase, and L-pyrrolidonyl-arylamidase and was identified by the system as *S. equinius*. An isolate taken from the nasal cavity of a healthy animal showed a positive reaction to Ala-Phe-Pro arylamidase and leucine arylamidase and was identified by the system as *S. pseudoporcinus* (Table 2).

**Table 2.** Biological properties of the *Streptococcus spp*. isolates under study

| Completed test | *S. agalactiae A* | *S. agalactiae B* | *S. anginosus* | *S. equinius* | *S. pseudoporcinus* |
|---|---|---|---|---|---|
| D-amygdalin | – | – | + | + | – |
| Phosphatidylinositol-phospholipase C | – | – | – | – | – |
| D-xylose | – | – | – | – | – |
| Arginine dihydrolase 1 | – | – | + | – | – |
| beta-Galactosidase | – | – | – | – | – |
| alpha-Glucosidase | + | + | – | – | – |
| Ala-Phe-Pro arylamidase | + | + | + | – | + |
| Cyclodestrin | – | – | – | + | – |
| L-aspartatarylamidase | – | – | – | – | – |
| beta-Galactopyranosidase | – | – | – | – | – |
| alpha-Mannosidase | – | – | – | – | – |
| Phosphatase | + | + | + | – | – |
| Leucinarylamidase | + | + | + | + | + |
| L-Prolinarylamidase | – | – | – | – | – |
| beta-Glucuronidase | – | – | – | – | – |
| alpha-Galactosidase | – | – | – | – | – |
| L-Pyrrolidonyl-arylamidase | – | – | – | + | – |

***Note:*** *"+" – 95-100% positive reaction; "–" – 0-5% positive reaction*

The isolates under study were cryopreserved and then lyophilized using two different media. For 6 months, freeze-dried crops were stored in the refrigerator at +2–+8°C.

Table 3 presents the results of revitalization of cultures after 6 months using pure meat-peptone broth before lyophilization without the addition of bovine blood serum. Therewith, the survival rate of isolates compared to the concentration of viable cells before lyophilization using pure meat-peptone broth was 0.16-1.07%, meat-peptone broth with the addition of 5% glucose – 0.46-1.68%, meat-peptone broth with the addition of 5% bovine blood serum – 0.33-3.89%, meat-peptone broth with the addition of 5% bovine blood serum and 5% glucose – 1.05-5.26%.

**Table 3.** The concentration of viable cells of *Streptococcus spp.*, restored with different nutrient media by lyophilization with pure meat-peptone broth

| Revitalization medium | Concentration of viable cells after lyophilization, CFU/cm$^3$ | | | | |
|---|---|---|---|---|---|
| | *S. agalactiae A* | *S. agalactiae B* | *S. anginosus* | *S. equinius* | *S. pseudoporcinus* |
| Meat-peptone broth | $1.7 \times 10^5$ | $2.4 \times 10^5$ | $7.9 \times 10^5$ | $5.8 \times 10^5$ | $3.0 \times 10^5$ |
| Meat-peptone broth + 5% bovine blood serum | $7.4 \times 10^5$ | $1.2 \times 10^6$ | $1.7 \times 10^6$ | $1.6 \times 10^6$ | $2.0 \times 10^6$ |
| Meat-peptone broth + 5% glucose | $3.2 \times 10^5$ | $4.9 \times 10^5$ | $8.1 \times 10^5$ | $7.9 \times 10^5$ | $6.1 \times 10^5$ |
| Meat-peptone broth + 5% glucose + 5% bovine blood serum | $1.0 \times 10^6$ | $1.6 \times 10^6$ | $2.1 \times 10^6$ | $1.8 \times 10^6$ | $2.1 \times 10^6$ |

Table 4 presents the results of revitalization using meat-peptone broth with the addition of 5% bovine blood serum before lyophilization. Therewith, the survival rate of isolates compared with the concentration of viable cells before lyophilization using pure meat-peptone broth was 0.25-1.04%, meat-peptone broth with the addition of 5% glucose – 0.36-2.47%, meat-peptone broth with the addition of 5% bovine blood serum – 1.05-4.68%, meat-peptone broth with the addition of 5% bovine blood serum and 5% glucose – 1.17-8.94%.

**Table 4**. The concentration of viable cells of *Streptococcus spp.* revitalized with different nutrient media by lyophilization with meat-peptone broth with the addition of 5% bovine blood serum

| Revitalization medium | Concentration of viable cultures after lyophilization, CFU/cm³ | | | | |
|---|---|---|---|---|---|
| | *S. agalactiae A* | *S. agalactiae B* | *S. anginosus* | *S. equinius* | *S. pseudoporcinus* |
| Meat-peptone broth | $1.8 \times 10^5$ | $5.1 \times 10^5$ | $1.0 \times 10^6$ | $7.7 \times 10^5$ | $4.5 \times 10^5$ |
| Meat-peptone broth + + 5% bovine blood serum | $8.9 \times 10^5$ | $1.3 \times 10^6$ | $1.9 \times 10^6$ | $1.8 \times 10^6$ | $2.1 \times 10^6$ |
| Meat-peptone broth + + 5% glucose | $4.7 \times 10^5$ | $5.5*10^5$ | $1.2*10^6$ | $1.1*10^6$ | $6.6*10^5$ |
| Meat-peptone broth + + 5% glucose + 5% bovine blood serum | $1.7 \times 10^6$ | $1.9 \times 10^6$ | $2.3 \times 10^6$ | $2.0 \times 10^6$ | $2.4 \times 10^6$ |

Cultures of *Streptococcus spp*. required special cultivation conditions and complex nutrient media containing blood or its serum. In turn, they were used for cryopreservation, lyophilization, and subsequent revitalization of these cultures.

Thus, in case of the cryopreservation procedure, the most effective was the preliminary cultivation of cultures of *Streptococcus spp*. with the addition of 5% bovine blood serum to the broth. All cultures that were lyophilized with the introduction of this nutrient medium showed a higher survival rate (0.25-8.94%) compared to the use of pure meat-peptone broth (0.16-5.26%) for revitalization in all combinations of nutrient media (Table 4).

Using various combinations of nutrient media to revitalize streptococci after lyophilization, the highest survival rates were obtained when using meat-peptone broth with the addition of 5% glucose and 5% bovine blood serum (1.17-8.94%) when lyophilizing meat-peptone broth with the addition of 5% bovine blood serum. Other combinations of nutrient media showed lower efficiency, in which the recovery rate was lower (0.25-5.26%). This phenomenon can be explained by the tenderness and fastidiousness of streptococcal cultures, in which the use of additives that enrich non-selective media with additional nutrients provides a higher probability of these cultures escaping sublimation. Therewith, the isolates under study stayed viable during the use of all combinations of nutrient media.

The results obtained coincide with the study of Ukrainian scientists Ushkalov *et al*. (2021), which determined the effectiveness of various nutrient media in revitalizing cultures after lyophilization. The composition and nutritional value of the media substantially affected the efficiency of revitalization of lyophilized cultures. At the same time, media with higher nutritional value were marked by a large amount of CFU during revitalization after lyophilization. It should also be noted that this study uses the most common nutrient media and components in laboratory practice, which increases the accessibility of this method.

The results of the study revealed some discrepancies with the study of Haindl *et al*. (2022). In this study, cultures of *Streptococcus spp*. almost did not alter their cultural and morphological properties after lyophilization and maintained their scientific and practical qualities even from the first reinoculation. This is primarily because unlike the research methodology of R. Haindl *et al*. (2022), in this study single isolates of alpha- and beta-haemolytic streptococci were used for lyophilization, rather than a pool of cultures.

The studies of Nasran *et al*. (2020) and Bodzen *et al*. (2021) indicated that an increase in the colloid of the liquid that is part of the cell walls of cultures contributes to a better preservation of the viability of these cultures after the lyophilization process, which is also followed in the presented study – the addition of glucose to nutrient media increased the resistance of microbial cells to lyophilization drying and ensured a higher percentage of their survival compared to combinations of environments in which glucose was not applied.

It is also important to note the studies of Oslan *et al*. (2017) and Bircher *et al*. (2018), in which the results of the study of different nutrient media for cryopreservation are presented. As in this study, it was shown that the use of a medium with added sugars contributed to better preservation of microorganisms during their freezing, which is explained by the lower destructive effect of water crystals on microbial cells.

Therewith, the studies of Boey *et al*. (2022) and Vekeman *et al*. (2013), which used various types of complex and simple sugars, found the destructive effect of complex sugars on bacterial cell walls. The established fact is explained by a violation of osmotic regulation of bacteria during binding to cell wall molecules. At the same time, the use of simple sugars, on the contrary, increased the resistance of bacteria to cryopreservation. This coincides with the results of the present study, where simple sugars were used for cryopreservation. With this combination of glucose-containing nutrient media, bacterial resistance, and survival after the lyophilization process were higher than using a combination of glucose-free nutrient media.

In the study of scientists Nicco *et al*. (2020) indicate the optimal temperatures for cryopreservation of bacteria using various freezing methods. At the same time, the presently described method in case of applying a temperature of -70°C was characterized by obtaining the same limits on culture survival that these scientists described in their research.

## Conclusions

The process of cryopreservation and subsequent lyophilization causes a considerable load on the microbial cell. In case of *Streptococcus spp*. cells, the greatest efficiency of cryopreservation and lyophilization, in which the survival of cultures was the highest, was obtained upon combination

of meat-peptone broth with the addition of 5% bovine blood serum with Faibich's medium 1:1 for cryopreservation and meat-peptone broth with the addition of 5% bovine blood serum and 5% glucose for revitalization of cultures after lyophilization.

The use of pure meat-peptone broth with Faibich's medium 1:1 compared to the use of meat-peptone broth with the addition of 5% bovine serum before lyophilization shows a lower percentage of survival of isolates of *Streptococcus spp*. using all combinations of revitalization media.

The use of other combinations of nutrient media for revitalization shows less efficiency but keeps a sufficient number of colony-forming units for further practical application of revitalized cultures.

To improve the efficiency of lyophilization and make it more accessible to revitalize cultures that are demanding on the composition of nutrient media and cultivation conditions, it is possible to use cheaper available nutrient media and components that are widely used in laboratory practice.

In the future, it is planned to continue research on identifying effective and practical methods for long-term storage and subsequent revitalization of alpha- and beta-haemolytic streptococci.

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# Ефективність поживних середовищ для відновлення ліофілізатів вибагливих мікроорганізмів на прикладі *Streptococcus spp.*

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**Анотація**. Для відновлення біологічних і морфологічних властивостей вибагливих бактерій при ліофілізації, одними з яких є вид стрептококи – бактерії роду *Streptococcus*, необхідно застосовувати дороговартісні спеціалізовані поживні середовища, які мають обмежену доступність для лабораторій. У зв'язку з цим, метою роботи було визначення найефективніших методів збереження та відновлення вибагливих мікроорганізмів на прикладі *Streptococcus spp*. Дослідження виконане бактеріологічним методом. Для дослідження відбиралися ізоляти *Streptococcus spp.*, які мали властивість до альфа- або бета-гемолізу. Збір мікроорганізмів проводили у результаті бактеріологічного дослідження патологічного і біологічного матеріалів від 20 тварин (10 собак і 10 котів). Визначення та підрахунок мікроорганізмів здійснено за допомогою систем Vitek 2 compact, easySpiral і Scan 500. У результаті проведених досліджень доведено ефективність застосування різних комбінацій поживних середовищ для кріоконсервації, ліофілізації та подальшого оживлення культур. Встановлено, що найефективнішим середовищем для ліофілізації є м'ясо-пептонний бульйон з додаванням 5 % сироватки крові великої рогатої худоби, розведений 1 : 1 з середовищем Файбіча, а для відновлення після ліофілізації – м'ясо-пептонний бульйон з додаванням 5 % сироватки крові великої рогатої худоби і 5 % глюкози. За такої комбінації концентрація життєздатних клітин відповідала межам від $1.7\times10^6$ до $2.4\times10^6$ КУО/см$^3$. Використання інших комбінацій поживних середовищ для оживлення бактерій роду *Streptococcus* виявляло меншу ефективність, що відповідало концентрації життєздатних клітин у діпазоні від $1.2\times10^5$ до $2.1\times10^6$ КУО/см$^3$. Отримані результати підвищують ефективність методу ліофілізації вибагливих культур завдяки використанню комбінації доступних у лабораторній практиці неселективних поживних середовищ і компонентів

**Ключові слова**: бактерія, кріоконсервація, ліофілізація, культуральне середовище, стрептокок