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The influence of nutrient medium on the reproduction of bacteriophages active against clinically significant microorganisms

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Abstract: *improving bacteriophage cultivation methods, optimizing nutrient media composition, and implementing modern production technologies will significantly increase the yield and quality of phage preparations. Enhancing production efficiency is a crucial step toward expanding the potential of modern phage therapy, raising the ability to combat antibiotic-resistant microorganisms, and stimulating the development of innovative biotechnological solutions. One of the key factors in improving the efficiency of phage production is the selection of optimal cultivation parameters. Changes in the chemical composition of the medium can either slow down or accelerate the growth rate of the bacterial culture, thereby indirectly affecting phage replication.*

To determine the impact of nutrient media type on bacteriophage reproduction, five different bacteriological media and nine bacteriophages active against nine species of clinically significant microorganisms were used. The effect of media composition was assessed based on the number and size of plaque formations. Optimal replication of all nine bacteriophages was observed on Nutrient Agar No 1, which contained peptic digest of animal tissue, meat extract, and yeast extract. Additionally, good phage reproduction was noted on Mueller-Hinton Agar and Nutrient Medium. The least suitable medium for phage cultivation was Tryptic Soy Agar, where bacteriophage replication was the lowest. Improving cultivation methods and optimizing the composition of nutrient media for bacteriophage reproduction are crucial steps toward enhancing the efficiency of their production and application in phage therapy.

Keywords: Bacteriophages; Reproduction; Phage Therapy; Biotechnology; Microbiology; phage-bacterial infection, nutrient agar, plaques.

Introduction

Bacteriophages (phages) are viruses that infect bacteria. They play a huge role in regulating bacterial populations and facilitating horizontal gene transfer [1]. Since their discovery in the early 20th century, bacteriophages have attracted researchers' attention due to their unique ability to specifically infect bacteria. In the context of the global crisis caused by antimicrobial resistance (AMR), interest in bacteriophages as an alternative or complement to antibiotics has significantly increased.

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Beyond medical applications, bacteriophages are actively studied in biotechnology, the food industry, and environmental sciences. They are used for pathogen biocontrol in food products, wastewater treatment, nanomaterial development, and more [2, 3]. Of particular interest is the use of bacteriophages in genetic engineering and synthetic biology to develop new therapeutic strategies and diagnostic platforms [4]. Despite numerous prospects, the clinical implementation of bacteriophages faces challenges such as production difficulties, regulatory barriers, and the need for standardization. While phage isolation in laboratory settings can be considered a routine process, scaling up these processes remains complex. Industrial enterprises producing phage-based preparations are primarily interested in reliable cultivation and production methods that enable large-scale manufacturing. The complexity of this task is mainly due to the biological nature of the systems and the diverse interactions between phages and bacteria [5]. At the same time, innovative approaches to optimizing phage cultivation and application processes open new horizons for their integration into modern medicine and industry. The reproduction of bacteriophages is closely linked to the physiological state of their bacterial host cells, which is largely determined by the cultivation medium. When grown on solid nutrient media, phages form distinct negative colonies or plaques. Plaque analysis remains an essential tool for studying various bacteriophages. Felix d'Hérelle, one of the founders of phage research, conducted the first plaque analysis as early as 1917 [6]. Over time, the plaque assay technique was improved by adding a layer of molten agar containing a mixture of phages and bacteria onto a pre-prepared layer of solidified agar medium. The molten agar solidified, immobilizing the phage particles within the semi-solid agar substrate. The molten agar solidified, embedding the phage particles in the semi-solid agar substrate. During incubation, the bacteria grew, forming a continuous bacterial layer, while the embedded phage particles destroyed bacteria in their vicinity, creating a zone of lysis on the bacterial surface [7, 8]. It is generally believed that each plaque in a solid nutrient medium originates from a single viral particle, although not all viral particles in the sample are capable of initiating an infection [9]. Anything that slows down the diffusion of phages can hinder plaque development and, consequently, affect their size. During plaque formation, the primary factors determining their size are the internal characteristics of phage diffusion within the medium, the effectiveness of the porous barriers for phage movement created by the cross-linked agar molecules, and the time phages spend infecting or interacting with the stationary bacteria [10]. However, experimental evidence has confirmed that temperature, medium composition, and bacterial feeding regimes have a greater impact on bacteriophage reproduction than other factors [11]. Optimization of phage production parameters (temperature and pH) allowed for a 70-fold increase in phage titer compared to the method where the culture was only shaken in a flask [12].

The aim of this study was to investigate the impact of different commercial nutrient media on the reproduction of bacteriophages with different morphologies, which are active against clinically significant microorganisms.

Materials and methods

Bacterial cultures: The study used nine reference strains of microorganisms obtained from the collections of ATCC and DSMZ: *Enterococcus faecalis* DSM 2570, *Escherichia coli* DSM 1103, *Proteus mirabilis* DSM 6674, *Pseudomonas aeruginosa* DSM 50071, *Staphylococcus aureus* DSM 346, *Enterobacter cloacae* DSM 6234, *Enterococcus faecium* DSM 2146, *Acinetobacter baumannii* DSM 30007, *Klebsiella pneumoniae* ATCC 700603.

Bacteriophages: Bacteriophages were isolated from wastewater by preliminary enrichment with appropriate host bacteria. Phage purification was carried out by three successive transfers from a single plaque. Specific activity was tested using the Gratia method and spot test. Phage morphology was studied using transmission electron microscopy (TEM).

Nutrient Media: To investigate the effect of different bacteriological media on the morphology and number of phage plaques, an analysis was conducted using various types of commercial

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media, specifically: Nutrient Agar No 1 (peptone enzymatic – 10.0 g/l; microbiological agar – 10.0 g/l; sodium chloride – 5.0 g/l; yeast extract – 3.0 g/l), Nutrient Medium (peptone enzymatic, dry for bacteriological purposes – 9.0 g/l; enzymatic hydrolysate of casein, low degree of hydrolysis – 8.0 g/l; yeast extract – 3.0 g/l; sodium chloride – 5.0 g/l; sodium phosphate – 2.5 g/l; microbiological agar – 13.0 g/l), Tryptic Soy Agar (pancreatic hydrolysate of casein – 15.0 g/l; papain hydrolysate of soybeans – 5.0 g/l; sodium chloride – 5.0 g/l; agar-agar – 15.0 g/l), Mueller-Hinton Agar (meat hydrolysate – 300.0 g/l; casein hydrolysate – 17.5 g/l; starch – 1.5 g/l; agar-agar – 17.0 g/l), Nutrient Agar No 2 (peptic digest of animal tissue – 5.0 g/l; meat extract – 1.5 g/l; yeast extract – 1.5 g/l; sodium chloride – 5.0 g/l; agar-agar – 15.0 g/l).

Experiments were conducted in triplicate, and statistical analysis was performed using the (mean value (±SD).

Results

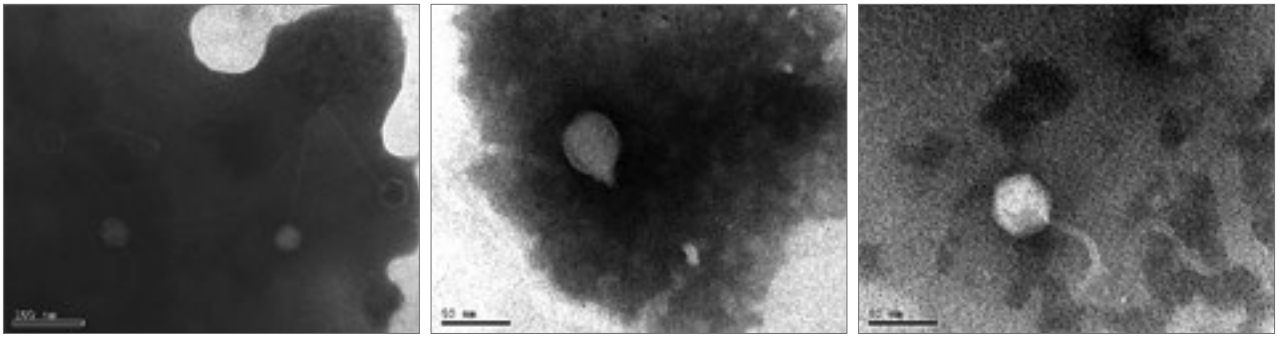
All bacteriophages used in the study were isolated from the municipal wastewater in Kyiv. Morphological analysis of the isolated phages using TEM revealed that they belong to different morphological groups. Accordingly, the study involved bacteriophages representing three main morphotypes: Siphoviridae (phages Enb2f, Enfs 14f, Enfm 1f, Kl178f, Pr48f, P1Sf), Podoviridae (phages Ac28f, St12f), and Myoviridae (phage E44f) – Fig. 1.

To determine the specific activity of the phages and the characteristics of the plaques formed by the isolated bacteriophages on different types of bacteriological media, the plaque assay method was used. In this process, 1 ml of overnight bacterial culture (depending on the species) was added to the corresponding nutrient medium, along with 100 µl of bacteriophage suspension, ensuring that between 30 and 300 plaque-forming units (PFUs) were formed on the surface and within the Agar. Since Tryptic Soy Agar (TSA) is most commonly used for isolating, describing, and cultivating bacteriophages, it was chosen as the control medium.

The results of the experimental studies showed that the phage plaques formed on various types of bacteriological media had distinct morphologies, ranging from very small to large plaques. These findings demonstrate significant variability in the reproductive activity of the bacteriophages depending on the type of nutrient medium used.

The maximum number of plaques formed by the Enb2f bacteriophage on various bacteriological media reached 70 PFUs per plate. No plaque formation was observed on TSA, whereas on Mueller Hinton Agar (MHA), 100 µl of the phage suspension resulted in an average of 25 PFUs per plate, with an average plaque size of 1.895±0.08 mm. On Nutrient Agar No.2, the number of plaques was 68 PFUs/ml, with an average plaque size of 2.175±0.47 mm, while on Nutrient Agar No.1, this value was 46 PFUs per plate, with an average plaque size of 1.885±0.27 mm. No plaque formation was observed on the Nutrient Medium.

The analysis of the effectiveness of the Enfs14f bacteriophage on various media showed



A **B** **C**

Fig. 1. Morphological features of the bacteriophages used in the study:
A – Bacteriophage Enfm 1f active against *E.faecium;* **B** – Bacteriophage Ac28f active against *A.baumannii*;
C – Bacteriophage Enb2f active against *E.cloacae.*

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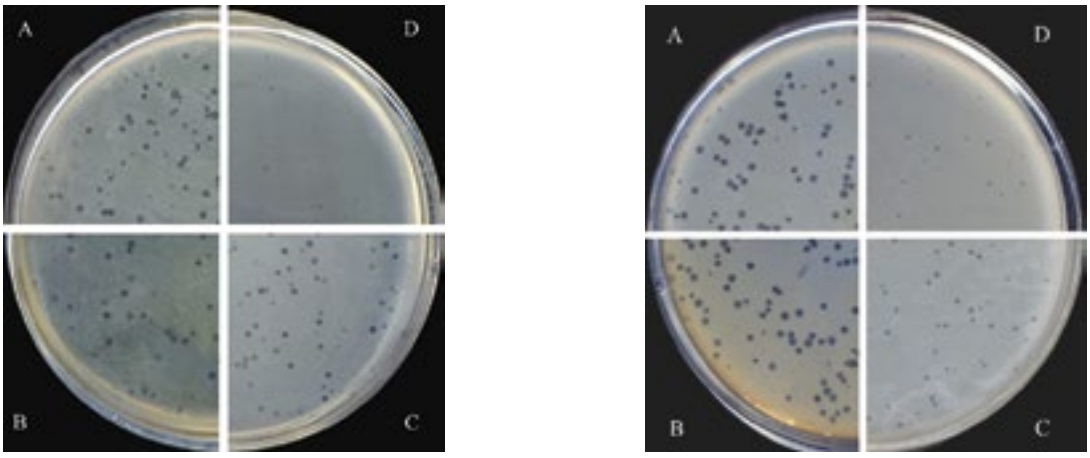
a significant increase in the number of plaques compared to TSA. Specifically, on Mueller Hinton Agar, the number of plaques was higher by 199% (P-value < 0.05), with an average plaque size of 1.025±0.07 mm compared to 0.92±0.07 mm (P-value < 0.05). On Nutrient Agar No.1, there was a 133% increase in the number of plaques (P-value ≥ 0.05), and the average plaque size was 1.025±0.07 mm and 1.045±0.05 mm, respectively (P-value < 0.05). The use of Nutrient Medium resulted in a 182% increase in the number of plaques compared to TSA (P-value < 0.05), with an average plaque size of 1.025±0.16 mm and 1.31±0.07 mm, respectively (P-value < 0.05). On Nutrient Agar No.2, there was a 216% increase in the number of phage plaques (P-value < 0.05), and the average size was 1.025±0.07 mm compared to 0.93±0.07 mm (P-value < 0.05).

The Enfm1f bacteriophages demonstrated a significant increase in plaque number on different media compared to TSA. On Mueller Hinton agar, there was a 173% increase (P-value < 0.05), and the average plaque size on TSA and MHA was 1.25±0.1 mm and 1.605±0.14 mm, respectively (P-value < 0.05). On Nutrient Agar No.1, the number of plaques increased by 160% (P-value < 0.05), and the plaque size was 1.25±0.1 mm on TSA and 2.055±0.11 mm on Nutrient Agar No.1 (P-value < 0.05). On Nutrient Medium, there was a 138% increase (P-value ≥ 0.05), with plaque sizes of 1.25±0.1 mm and 1.96±0.2 mm on TSA and Nutrient Medium, respectively (P-value < 0.05). Finally, on Nutrient Agar No.2, the number of plaques increased by 177% (P-value < 0.05), and the plaque size on TSA and Nutrient Agar No.2 was 1.25±0.1 mm and 2.07±0.19 mm, respectively (P-value < 0.05) (Fig 2).

Kl178f phages showed similar trends, with increased plaque numbers on various media. On Mueller Hinton agar, the number of plaques increased by 168% (P-value < 0.05), and the average plaque size was 0.94±0.05 mm on TSA and 1.605±0.14 mm on MHA (P-value < 0.05). On Nutrient Agar No.1, the number of plaques increased by 218% (P-value < 0.05), with plaque sizes of 0.94±0.05 mm on TSA and 1.56±0.05 mm on Nutrient Agar No.1 (P-value < 0.05). On Nutrient Medium, the number of plaques increased by 274% (P-value < 0.05), with plaque sizes of 0.94±0.05 mm and 1.8±0.06 mm, respectively (P-value < 0.05). Finally, on Nutrient Agar No.2, the number of plaques increased by 266% (P-value < 0.05), and the average plaque size was 0.94±0.05 mm on TSA and 1.885±0.13 mm on Nutrient Agar No.2 (P-value < 0.05) (Fig 2).

The Pr48f phages showed no significant difference in the number of plaques across TSA, Mueller Hinton agar, and Nutrient Agar No.1. The plaque sizes also did not show statistically significant differences. A slight increase in the



A **B**

Fig. 2. The effect of different types of bacteriological media on bacteriophage growth:
A – phage Enfm 1f (bacterial culture – *E. faecium* DSM 2146), **B** – phage Kl178f (bacterial culture – *K. pneumoniae* ATCC 700603). A – Nutrient Agar No. 2; B – Nutrient Medium; C – Mueller Hinton Agar; D – TSA.

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number of plaques was observed on Nutrient Medium (P-value ≥ 0.05). However, on Nutrient Agar No.2, the number of plaques more than doubled (P-value < 0.05), while the plaque size remained unchanged.

P1Sf phages demonstrated an 8% reduction in the number of plaques on MHA compared to TSA (P-value ≥ 0.05). The plaque size did not show statistically significant differences between the two media, with sizes of 1.975±0.14 mm on TSA and 2.045±0.16 mm on MHA (P-value ≥ 0.05). On Nutrient Agar No.1, the number of plaques increased by 118% compared to TSA (P-value ≥ 0.05), with plaque sizes remaining similar at 1.975±0.14 mm on TSA and 2.0±0.34 mm on Nutrient Agar No.1 (P-value ≥ 0.05). On Nutrient Medium, there was a 123% increase in the number of plaques (P-value < 0.05), and the plaque size was 1.975±0.14 mm on TSA and 2.09±0.23 mm on Nutrient Medium (P-value ≥ 0.05). Finally, on Nutrient Agar No.2, the number of plaques increased by 133% compared to TSA (P-value < 0.05), and the plaque size did not change, measuring 1.975±0.14 mm on TSA and 2.125±0.32 mm on Nutrient Agar No.2 (P-value ≥ 0.05).

For Ac28f phages, the number of plaques on MHA increased by 141% compared to TSA (P-value < 0.05), with the plaque size remaining similar at 2.215±0.5 mm on TSA and 2.515±0.7 mm on MHA (P-value ≥ 0.05). On Nutrient Agar No.1, the number of plaques increased by 160% compared to TSA (P-value < 0.05), and the plaque size increased from 2.215±0.5 mm on TSA to 2.6±0.6 mm on Nutrient Agar No.1 (P-value < 0.05). On Nutrient Medium, the number of plaques remained unchanged compared to TSA (P-value ≥ 0.05), while the plaque size increased from 2.215±0.5 mm on TSA to 2.86±0.7 mm on Nutrient Medium (P-value < 0.05). Finally, on Nutrient Agar No.2, the number of plaques increased by 295% compared to TSA (P-value < 0.05), with the plaque size increasing from 2.215±0.5 mm on TSA to 2.63±0.5 mm on Nutrient Agar No.2 (P-value < 0.05).

For phages St12f, the number of plaques on TSA, Mueller Hinton agar, Nutrient Agar No 1, and Nutrient Medium did not differ. On Nutrient Agar No 2, the number of plaques increased by 119% (P-value ≥ 0.05), while the size of the plaques on TSA and other media was as follows: 0.84±0.07 on TSA, 0.935±0.1 on Nutrient Agar No 1 (P-value < 0.05), 1.13±0.1 on Nutrient Medium (P-value < 0.05), and 1.09±0.13 on Nutrient Agar No 2 (P-value < 0.05).

Phages E44f showed a significant increase in the number of plaques on MHA by 190% compared to TSA (P-value < 0.05), with plaque sizes on TSA and MHA being 0.92±0.04 and 1.08±0.04, respectively (P-value < 0.05). On Nutrient Agar No 1, the number of plaques increased by 272% compared to TSA (P-value < 0.05), with plaque sizes on TSA and Nutrient Agar No 1 being 0.92±0.04 and 0.98±0.04, respectively (P-value < 0.05). On Nutrient Medium, the number of plaques increased by 180% compared to TSA (P-value < 0.05), and plaque sizes on TSA and Nutrient Medium were 0.92±0.04 and 1.075±0.4, respectively (P-value < 0.05). On Nutrient Agar No 2, the number of plaques increased by 318% compared to TSA (P-value < 0.05), and the plaque sizes on TSA and Nutrient Agar No 2 were 0.92±0.04 and 1.085±0.03, respectively (P-value < 0.05).

Discussion

Studying the influence of nutrient media on bacteriophage reproduction is of great importance both for fundamental virology (bacteriophageology) and for the practical use of bacteriophages in phage therapy of bacterial infections. The results of such studies allow for the optimization of conditions for phage cultivation, which is essential for their industrial production and laboratory research. In this study, the impact of different bacterial media compositions on bacteriophage reproduction was investigated, which was assessed by the number of plaques formed on the nutrient medium and their size. According to the obtained results, significant variability in the reproductive activity of phages was observed depending on the type of medium. The bacteriophages used in the experiment showed considerable differences in plaque sizes on different types of media. Preliminary studies conducted by various researchers suggest that the plaque size is directly proportional to the burst size (the

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number of newly produced phages in the cell), the phage adsorption constant, and their diffusion in the medium, as well as inversely proportional to the latent period, with each of these factors contributing its part [13]. In contrast, it has also been experimentally confirmed that the rate of phage production is proportional to the amount of the protein synthesis machinery per cell at the moment of infection and is not limited by the cell size or DNA composition. Larger and faster-growing cells contain proportionally more protein synthesis systems, which leads to increased phage production [14]. Thus, the presence of necessary components in the nutrient medium to enhance the growth rate of certain microorganism species may lead to an increased phage reproduction, which, in turn, will affect the size of the phage plaques. In the course of the experimental study, it was found that for most phages, an increase in the number of plaques was observed when using media such as MHA and Nutrient Agar No. 2, compared to the traditional TSA. Both types of media contained meat hydrolysate, which is a good substrate for the growth of many microorganisms. The increase in the number of plaques on alternative media compared to TSA was statistically significant for most phages. The obtained results align with other studies where different types of media were investigated. For example, Muhammad Kamran Taj et al. demonstrated that nutrient media containing a high amount of organic substances better support phage reproduction compared to poor media [15]. The influence of different types of nutrient media on phage reproduction was also demonstrated using bacteriophages active against *Staphylococcus aureus* and *Vibrio parahaemolyticus* [16].

It is important to note that the study was conducted on a limited number of phages and bacterial strains. This means that the results may not be universal for all bacteriophages. Additionally, other potential factors that were not the focus of this study should be considered.

From the above results, it can be concluded that the composition of bacteriological media has a significant impact on the phenomenon of plaque formation, demonstrating that plaques formed by a single bacteriophage differ in number and size across different media. Further research could include expanding the spectrum of phages and bacterial hosts, as well as investigating the impact of different media components on phage reproduction processes. The choice of optimal medium is critical for the further use of bacteriophages in research and practical applications, such as therapy or biocontrol of pathogenic microorganisms.

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Conflict of interests

The authors declare no competing interests.

Consent to publication

Not applicable.

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A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of article.

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Вплив поживного середовища на репродукцію бактеріофагів, активних проти клінічно значущих мікроорганізмів

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Анотація: *вдосконалення методів культивування фагів, оптимізація складу поживних середовищ та впровадження сучасних технологій їх виробництва дозволять значно збільшити вихід та якість фагових препаратів. Підвищення ефективності виробництва є важливим кроком для розширення можливостей сучасної фаготерапії, збільшення можливостей щодо боротьби із антибіотикорезистентними мікроорганізмами і стимулювання розвитку*

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інноваційних біотехнологічних рішень. Одним із важливих елементів щодо підвищення ефективності виробництва фагів є підбір оптимальних параметрів щодо їх культивування. Зміни в хімічному складі середовища можуть уповільнювати або прискорювати швидкість розмноження бактеріальної культури, таким чином опосередковано впливати на репродукцію фагів.

Для визначення впливу типу поживних середовищ на репродукцію бактеріофагів використано 5 різних за складом бактеріологічних середовищ та 9 різних бактеріофагів, що активні у відношенні до 9 видів клінічно-значимих мікроорганізмів. Вплив складу середовища визначали за кількістю утворених бляшок та їх розміром.

Оптимальна реплікація всіх 9 бактеріофагів спостерігалася на середовищі Nutrient agar № 2, що містило пептичний перевар тваринної тканини, м'ясний екстракт і дріжджовий екстракт. Крім того, хороша репродукція використаних в дослідах бактеріофагів була на середовищах Mueller Hinton agar та на Nutrient agar № 1. Найменш придатним для культивування фагів виявився Tryptic Soy agar, репродукція фагів на даному типі середовища була найнижчою.

Вдосконалення методів культивування та оптимізація складу поживних середовищ для культивування бактеріофагів є важливим кроком для підвищення ефективності їх виробництва та застосування у фаготерапії.

Ключові слова: Бактеріофаги, бляшки, поживні середовища, репродукція, фагово-бактеріальна інфекція.