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Low-temperature drying: experimental viability analysis of bacteriophages (*Siphoviridae family*) immobilized in alginate substrate.

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Abstract

Supplementing animal food with bacteriophages active against animal bacterial pathogens can be an excellent way of mildly influencing the quality of food and the health condition of animal breeding. Polysaccharides were used to immobilize biologically active cultures, and the technique of low-temperature drying under atmospheric pressure was chosen to maintain microbiological activity. Periodically dried material had the form of spheres with initial diameter 3 mm and moisture content 97%.

The average initial concentration of bacteriophages of Siphoviridae family was 1×10^8 PFU/g (plaque-forming units per gram).

Drying tests were carried out in temperatures range from -10°C up to 30°C. The activity of bacteriophages was checked prior and after drying, by enumeration by double agar overlay plaque assay.

Keywords: *low-temperature drying, drum drying, bacteriophages, the activity of microorganisms.*

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1. Introduction

One of the major breakthroughs in the history of medicine is undoubtedly the discovery of antibiotics. Antimicrobial drugs have been successfully and widely used in human and veterinary medicine for more than 60 years [1]. Almost simultaneously, the use of antimicrobials has been applied in agriculture. Their use in animal husbandry and veterinary medicine has resulted in healthier and more productive farm animals, ensuring the welfare and health of both animals and humans [2, 3, 4].

Bacteria are quite adaptive organisms that have survived multiple environmental stresses during their existence on the planet. Thus the emergence of antibiotic-resistant bacteria occurred quite shortly after their first use [5]. Antimicrobial resistance is a national and worldwide public health challenge. The drug resistance and legislative restrictions on the use of antibiotics in livestock farming in the countries of the European Union, [6, 7, 8] and in the USA [1] make it necessary to develop new solutions in the elimination of pathogens. In recent years a number of scientists and clinicians are looking again at application of bacteriophages as a therapeutic option in the treatment of bacterial pathogens infections that may contaminate the food supply [9, 10]. Bacteriophages are viruses that infect bacteria. Like any virus, they are parasites, requiring the host cellular system to reproduce. Infection begins by attachment of the phage particle to its host cell through specific recognition of a receptor on the host surface, followed by delivery of the phage nucleic acids into the infected cell. At this stage lytic bacteriophages genetic material hijacks host cell replication mechanism, leading to formation of new phage particles that are subsequently released, destroying bacterial cell in the process [11]. The International Committee on the Taxonomy of Viruses (ICTV) recognizes 1 order, 13 families, and 31 genera of bacteriophages. Bacteriophages have four basic shapes or symmetries [12]. Most of the characterized phages are 'tailed phages' [13], which have double-stranded DNA (dsDNA). These phages are classified into three families: *Myoviridae*, *Siphoviridae* and *Podoviridae*. The rest of the phages, constituting only 4% of the total, are classified into 10 families [14]. In this study phage cocktail from *Siphoviridae* family were used as a preventive measure against Salmonella - gram-negative, rod-shaped bacterium that belongs to the family of *Enterobacteriaceae*. Polysaccharides of natural origin were used to immobilize the biologically active material. The first of them was alginate which is a naturally occurring biopolymer that is finding increasing applications in the biotechnology industry. Alginate has been used successfully for many years in the food and beverage industry as a thickening agent, a gelling agent and a colloidal stabilizer. Alginate also has several unique properties that have enabled it to be used as a matrix for the entrapment and/or delivery of a variety of proteins and cells [15]. Alginate was doped with another natural and hydrophilic copolymer. Due to "know-how" agreement in this article we cannot provide the name of this substance. Similarly to Alginate, the second polysaccharide has been approved for medical, pharmaceutical and food applications due to its biocompatibility, biodegradability, non-toxicity and non-carcinogenicity [16]. The obtained cross-linking of these two copolymers are characterized by enhanced stability in comparison with those obtained with a single polymer. Moreover this addition in the formulation caused an increase of the mechanical resistance of the bead structure and as a consequence, a limitation of the bead swelling ability.

A stiff and brittle copolymer spheres with a predominance of α-L-guluronic acid monomers was obtained, which without the drying process underwent the phenomenon of syneresis - shrinkage with release of moisture. Low-temperature drying under atmospheric pressure was used, alternative method of producing microbiologically active additives in animal feed. As the comparative method lyophilization was applied, which is a commonly used technique for drying sensitive materials.

2. Materials and Methods

2.1. Preparation of alginate beads

Beads were prepared by dissolving sodium alginate (Biomus sp. z o.o., Poland) in bacteriophage preparation (Proteon Pharmaceuticals S.A., Poland) under mechanical stirring. Alginate gel was then extruded into solution of divalent ions and polysaccharide polimerizing agent and stirred until beads hardened. Beads were washed with water to remove any excess hardening solution. Raw material was presented in the Fig. 1.

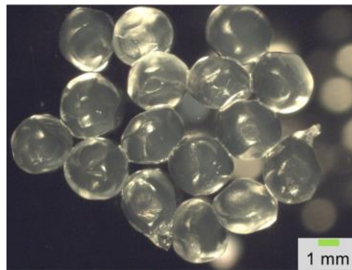

Fig. 1 Doped alginate sphere before drying.

2.2. Drying

Due to the size of doped alginates - spheres with initial diameter of about 3 mm - four different drying methods were considered: lyophilisation, fluidization, fixed bed and in a drum dryer. The second method was rejected due to the fragility and dustiness of the material after drying, according to preliminary experimental work. Low solids content (initial moisture content above 97%), suggests that despite intensification of the process during fluidization, significant abrasion and shrinkage of drying material will occur. During preliminary experiments in a fixed bed depending on the weight of the charge, non-uniformly drying of the material was noticed, therefore it was ultimately decided to carrying out processes in the drum dryer. Experiments in drum dryer presented in this paper were carried out in atmospheric freeze drying system built in the Department of Heat and Mass Transfer, Lodz University of Technology. The experimental rig worked in a closed-loop system which eliminated emissions of dusts and odors to the atmosphere or from the atmosphere to drying material. The installation is presented schematically in Fig. 2.

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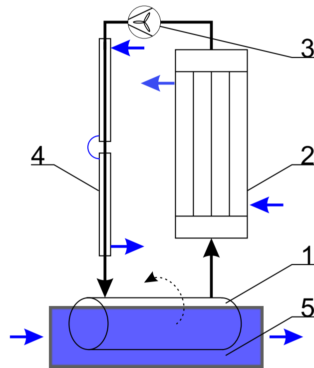

Fig. 2 Schematic view of drying installation. where: 1 – drying chamber, 2 – dehumidifier, 3 – fan, 4 – second heat exchanger, 5 – glycol thermostatic bath.

The dried material was placed in a cylindrical chamber with partitions and total volume 2 dm^3. During all processes, the rotational frequency was 15 rpm. In order to determine the drying kinetics, the container with dried material was removed from the installation, sealed and manually weighed on a laboratory balance. At the initial stage of the process, the material could stick to the walls of the drying chamber due to the high content of surface moisture, for this reason, after each weighing intensive shaking was made to peel off material from the drying chamber walls. After leaving the drying chamber, gas is directed to the heat exchanger where the drying agent is cooled and moisture is removed in the form of mist or frost. Cooled and dry gas is directed through the fan to the heat exchangers, where the gas is adapted to predetermined process parameters. So prepared drying agent flows to the drying chamber and the cycle begins again. During the drying process, the drying chamber was thermostated in a glycol bath to eliminate the effect of ambient temperature on the process. As the comparative method lyophilization was performed on Christ Alpha 2-4. Before lyophilization, material was stored for 12 hours in a freezer at -28°C. Lyophilization process was carried out for 6 hours at -25°C and pressure 63 Pa, then the temperature was raised to 30°C, pressure reduced to 3,5 Pa for the next 10 hours.

2.3. Dried material structure

After each test, samples of dried material were collected for microbiological and morphological analysis. Microscopic images of particles were obtained applying Nikon SMZ 1000 stereoscopic microscope equipped with Lumenera Infinity 1 digital camera.

2.4. Enumeration of bacteriophages encapsulated in beads

The activity of bacteriophages was checked prior and after drying and after 8-week storage. Double agar overlay plaque assay is considered “golden standard” in microbiology [17] and the method was applied to assess the number of active phage particles in each sample. Beads were dissolved by shaking (140 rpm, 37°C, 1 hour) in phosphates solution (POCH, Poland). For each sample, three independent series of dilutions were prepared. 100 µl of overnight host bacterial culture and an aliquot of each phage dilution was transferred to the pre-warmed (54°C) test tubes with 6 ml of 0,7% solution of agar in LB medium. The suspension of phages and cells in agar was then mixed by vortexing, poured on to agar

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plates containing solid medium and swirled until top agar layer had evenly covered the bottom layer. The plates were incubated overnight in at 37°C to let bacteria overgrow the agar medium and form a bacterial lawn. Clear spots in the bacterial lawn (plaques), where phage particles infected and lysed bacterial cells, were counted. PFU (plaque forming units) per 1 gram of beads (PFU/g) was calculated according to the following equation:

$$T_{ph} = \frac{\sum c}{V_p \times (d_1 \times N_1 + d_2 \times N_2 + d_3 \times N_3)} \times \frac{V_s}{m_b}$$

where:

Tph – bacteriophage titer (PFU/g),
c – the sum of all plaques on plates with three consecutive dillutions,
V_p – a volume of phage suspension poured on each plate,
d_{1-3} – dilutions poured on plates,
N_{1-3} – number of plates with the corresponding dilution,
V_s – a volume of dissolvent,
m_b –mass of beads being dissolved.

The method was validated in Proteon Pharmaceuticals SA in accordance with Good Laboratory Practice (GLP) quality management system procedures. Results were only accepted if they met the criteria specified in validation protocol (data not shown).

3. Results and discussion

3.1. Drying kinetics

Fig. 3 shows the drying curves for the different drying air temperatures (-10, 0, 10, 20, 30°C). As it can be seen in these figures, drying kinetics within the range of the average moisture contents considered, showed the existence of only one constant rate period. A clear effect of the temperature on the drying curves of the doped alginate spheres can be observed. An increase of the drying temperature is accompanied by a decrease on the drying time.

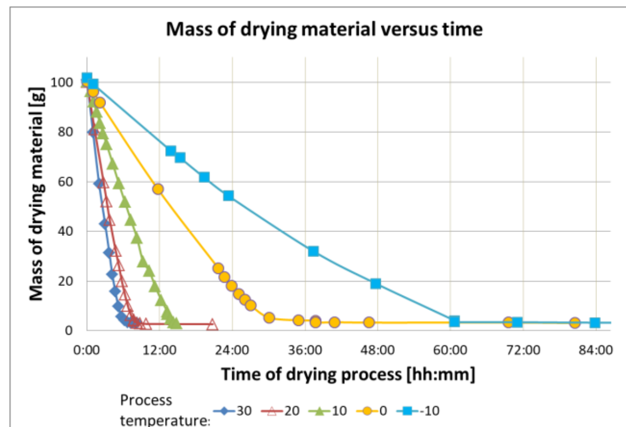

Fig. 3 Mass of dried alginate spheres versus process time.

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3.2. Effectiveness of drying process in relation to bacteriophage activity and 8-week stability of dried samples.

After the drying process, bacteriophages were characterized by high activity. The highest value has been noted for the process temperature + 10°C. The lowest for -10°C and for the lyophilization process. All obtained results: effectiveness after process and stability after 8 weeks storage is presented in Fig. 4. Effectiveness was calculated as PFU/g after drying relative to PFU/g prior drying process. Stability was calculated as PFU/g after 8-week storage in 4°C relative to PFU/g after the drying process.

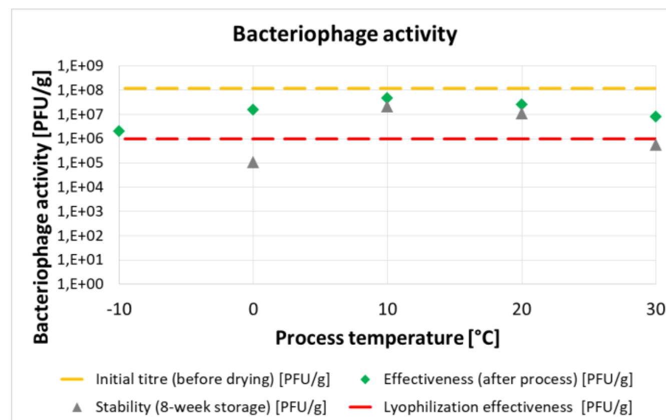
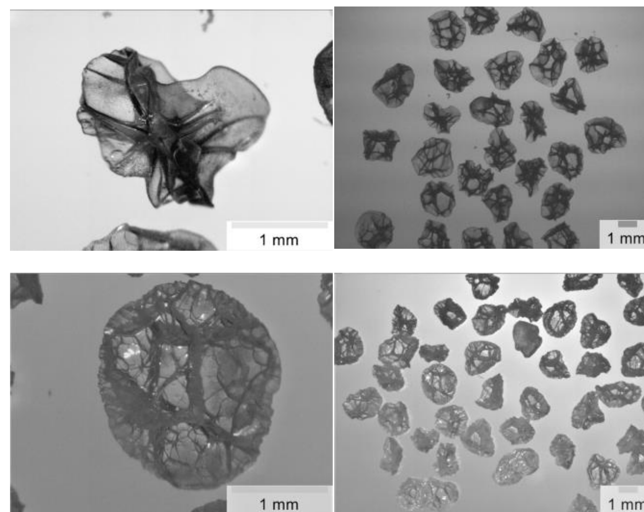


Fig.4 Bacteriophage activity versus drying parameters.

Samples were stored 8 weeks at 4°C and then activity was measured again. Bacteriophage activity of sample dry at -10°C dropped below the detectable level.

3.3. Dried material structure

Depending on the process parameters, three different types of dried material structures were obtained. The microscopic images of these structures are presented in Fig. 5.



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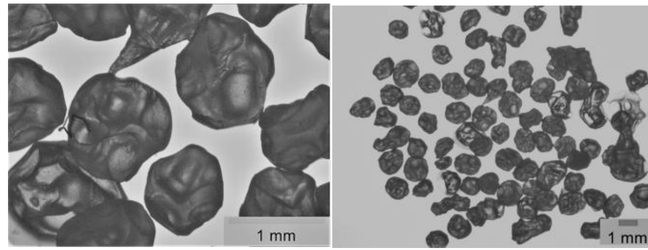

Fig.5 Microscopic images of dry particles, from the top: lyophilization, -10°C, above 0°C processes.

After the drying process at -10°C, dust derived from the dried material was observed inside the drying installation. It was concluded that during drying processes at low temperatures it is necessary to gradually reduce the rpm of the drying chamber in order to reduce the adverse phenomenon of material abrasion. Samples dried at temperatures above 0°C were characterized by a globular shape, with a compact structure. No abrasion phenomenon was observed during these processes. Low-temperature dried material, i.e. <0°C, was characterized by the lowest drying shrinkage and a well-developed surface area of the particle. Such process parameters had the most beneficial effect on the carrier of the biologically active material. The expanded surface area for the material with a biologically active component is preferably a desirable phenomenon affecting the efficiency of the transfer of compounds into the organism.

4. Conclusions

This preliminary study serves as a proof of concept that alginate-encapsulated bacteriophages retained their ability to infect host bacterial strain after all drying processes conducted in temperature equal or higher than 0°C. The highest bacteriophage activity after drying as well as after 8 weeks storage at 4°C was obtained for the material dried in 10 Celsius degree. This activity was higher than for the low-temperature process conducted below 0°C and the lyophilization process. The results indicate an adverse effect of negative temperatures on bacteriophages. To test this hypothesis, samples with fresh material were stored for a period of 4 days (assumed duration of the process) at temperatures: -23, -15, -10, -5, -1°C. Then the material was thawed and the activities analyzed. The effect of low temperatures on bacteriophage activity was not noticed. However, after 8 weeks of storage at 4°C after low temperature drying processes according to the presented results, the bacteriophage titer was below the limit of quantification. In our opinion, the low temperature in the process weakens the bacteriophage structure, which requires further research.

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