Study of freeze-drying process of bacterial concentrates in the apparatus using thermoelectric modules

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Abstract. The method of lyophilization is the only possible way to preserve starters, microbiological cultures, probiotics, enzyme preparations, hormones, vaccines, antibiotics, enzymes and other biological materials. An essential aspect of the implementation of the lyophilization method is the preservation of the biological activity of materials. In the case of bacterial concentrates, this indicator is estimated by the number of viable cells at the end of the dehydration process and subsequent storage. Freezing is the first and most important stage of lyophilisation, during which the final product structure is formed, affecting the quality of the material. Freezing rate and temperature are determined individually for each preparation in order to select conditions ensuring maximum safety of product structural integrity. The purpose of researches is a complex definition of reasonable intervals of parameters of freeze-drying of bacterial concentrates that will allow optimizing this technological process. Criteria of optimization are qualitative parameters of the product and energy consumption for freeze-drying. Also, a comparative analysis of the energy efficiency of the developed freeze-dryer and its existing analogues has been performed. Each of these substances has a specific eutectic point, which leads to a eutectic zone of concentrates. Thus, the freezing temperature of bacterial concentrates should be several degrees below the upper eutectic point, i.e. in the range of minus 39 to minus 42 °C. During the freeze-drying, the following rational parameters were established: residual pressure in the chamber 0.05-0.06 mbar, the final temperature of the material 34-35 °C, the temperature on the condenser surface minus 55 °C.

1. Introduction

Lyophilic (freeze-drying) currently has no alternative for the ability to preserve the labile components of biological products in natural form. The method of lyophilization is the only possible way to preserve starters, microbiological cultures, probiotics, enzyme preparations, hormones, vaccines, antibiotics, enzymes and other biological materials. The unquestionable advantages of lyophilization include the possibility of long-term storage of the received preparations (under the condition of hermetic packing) with the subsequent fast restoration of properties at hydration, low weight, structure preservation.

An important aspect of the implementation of the lyophilization method is the preservation of the biological activity of materials. In the case of bacterial concentrates, this indicator is estimated by the...
number of viable cells at the end of the dehydration process and subsequent storage. From the concept of «functional nutrition» in fermented milk products, the presence of live cultures of probiotics is mandatory, due to which the peristalsis of the gastrointestinal tract, microbiocenosis, immunity, and metabolism in conditions of unstable ecological situation improve [1].

The production of products with probiotic orientation is based on the use of starter cultures of microorganisms, which are introduced in the form of bacterial ferments and concentrates. A bacterial concentrate is biomass in which the number of cells is two to three orders of magnitude higher than in starter. This fact makes it possible to intensify the process of squeezing, to reduce the production area and to increase the sanitary and hygienic indices of the product [2]. Lyophilization includes pre-freezing of the material necessary for the transition of moisture into a solid aggregate state, and further freezing of the ice at a deep vacuum in the chamber of the apparatus (residual pressure is below the «triple point» for water, equal to 6.12 mbar).

The energy-saving trend is a worldwide trend for all industries. In lyophilic drying, the primary energy costs are related to the supply of energy to the material to be dried to ensure the phase transition of ice vapour, the operation of a refrigeration unit designed to reduce the temperature on the condenser surface (desalinator) and the vacuum system. Observance of technological parameters of the pre-freezing process and the subsequent lyophilization is a vital condition affecting not only the quality of the final product but also energy costs at all stages.

2. The purpose of the study
Freezing is the first and most important stage of lyophilisation, during which the final product structure is formed, affecting the quality of the material. Freezing rate and temperature are determined individually for each preparation in order to select conditions ensuring maximum safety of product structural integrity [3].

During freezing the following factors must be taken into account: process speed, presence of cryoprotective medium and eutectic material temperature value. At the low freezing rate, large crystals will form in the sample, and at high speed, small crystals will form in the sample. The survival rate of microorganisms can be increased by freezing with nitrogen vapour or in liquid nitrogen media. At this rate of freezing water does not have time to leave the cell, which reduces its dehydration, the structure of the ice becomes fine crystalline, the time of action of hyper-concentrated solutions of salts decreases. Exposure to fine ice crystals does not cause cell death.

Cryoprotective media are used for increasing microbial survival during freezing. They are substances that can prevent the development of cryoprotective damage to biological objects and ensure their viability after freezing. Thus microorganisms are in the state of anabiosis, which increases their resistance to low temperatures.

As cryoprotectants can be used: proteins (gelatin, albumin), carbohydrates (glucose, fructose, lactose, sucrose), amino acids (alanine, valine, glycine), inorganic salts (NaCl, MgCl2), skim milk powder [4].

The eutectic temperature is the temperature of complete crystallisation of the solution. The liquid in the product and the solid phase formed during freezing are in equilibrium. Bacterial concentrates are liquid biomaterial containing organic and mineral substances. Therefore they are characterized by a eutectic temperature zone, which can be from 8 degrees and more [5]. Compliance with the freezing regime will prevent local melting of ice during lyophilization and, consequently, increase the number of viable cells.

During dehydration of frozen biomass, a whole complex of phenomena influencing microbial survival occurs. The main causes of cell death are protein denaturation and enzyme inactivation. The need to preserve proteins is due to their key role in the normal vital functions of microorganisms. Protein denaturation can occur as a result of an increase in the concentration of mineral substances in the cell when moisture is removed. Another reason for microorganisms death directly at lyophilization or at further storage is an increase in the ultra-permissible concentration of toxic components.
Justification of the possibility to increase the efficiency of the lyophilization process due to the application of new technological and technical solutions is given in the works of domestic and foreign researchers [6, 7]. The main technological parameters include the rate of freezing of the concentrate, the temperature of freezing and sublimation of the material, the density of heat flow of power supply sources, the degree of rarefaction in the chamber, the temperature on the surface of the condenser (desublimation).

Requirements for the technical equipment of lyophilic equipment are dictated by Good Manufacturing Practice (GMP) standard and are constantly tightening. These circumstances make it possible to achieve full process validation, increase the degree of automation, and reduce personnel interference in sterile production.

When designing lyophilic equipment that meets the criteria of the GMP standard, the following principles are used:

- achieving an even heat supply to the tanks with biological material,
- the use of secondary heat resources (Peltier effect, Rank effect) to improve the energy efficiency of equipment, intensifying the supply of energy needed for sublimation to the front of the phase transition of ice pairs.

According to the Green Peace organization, lyophilic drying is the leader in terms of ecology among traditional methods of dewatering, since in this process the most environmentally friendly sources of heat (infrared lamps, microwave and microwave) and energy (electric current) are used [8]. However, to obtain low temperatures on the surface of the desublimation during freezing of water vapours removed from the material, steam-compression refrigeration units are used. Freons are used as refrigerants, many of which are ozone-depleting.

Thermoelectric elements, being one of the most promising in the creation of environmentally friendly cooling devices, are increasingly winning the international market as an alternative to traditional steam-compression and absorption equipment. Advantages of thermoelectric cooling devices are environmental cleanliness, no refrigerants, small size, noiselessness, average operating time on failure not less than 200 thousand hours, independence from orientation in space, change of cooling and heating modes due to current reversal. We have developed a lyophilic unit in which design is described in [1, 9].

The purpose of researches is a complex definition of reasonable intervals of parameters of the lyophilization process of bacterial concentrates that will allow optimizing this technological process. Criteria of optimization are qualitative parameters of the product, degree of automation of equipment and energy consumption for lyophilic drying. Besides, a comparative analysis of the energy efficiency of the developed lyophilic dryer and its existing analogues has been performed.

3. The object of the study
The objects of research at all stages of the experiments were: a bacterial concentrate of mesophilic lactic acid bacteria *L. casei* and bifidobacteria *B. adolescentis*.

4. Materials and methods
During the experiments the following materials were used: distilled water (GOST 6709-72); nutrient medium Lee (GOST R 54065-2010); nutrient medium for lactic acid bacteria (GOST 10444.11-89); skimmed milk (GOST 31658-2012); yeast extract (GOST 30134-97); sucrose (GOST 5833-75); gelatine (GOST 11293-89); sodium citrate (GOST 31227-2013); table salt (GOST R 51574-2000); phosphate buffer with pH 7.2 (GOST 9225).

In the course of the research, the equipment of the «BioTech» laboratory Centre of collective use «Control and management of energy-efficient projects» of VSUET was used: synchronous thermal analyzer STA 449 F3 (TGA/DSC) (P 57952-2017), which allows determining the values of temperature and heat transitions by the method of differential scanning calorimetry, lyophilic dryer LS 1000K «Prointech», low-temperature freezer HAIER DW 8, sterilization module by autoclaving with automatic control unit SPVA 75-1 HN, pH-meter Edge HI 2002-02, analytical electronic scales AF-
R220E, electronic scales AJ-1200CE, low-temperature laboratory electric furnace SNOL 24/200, moisture meter FD-610, a box of abacterial air environment II class BAVp-0,1-Laminar C 1,2 (221).120).

For achieving this goal, standard methods of investigation (microbiological, chemical and physical-chemical) were used.

4.1 Study of eutectic temperatures of bacterial concentrates
During the experiments, the biomass of mesophilic lactic acid bacteria and bifidobacteria were separated after cultivation. The cryoprotective medium was injected with the following composition: gelatine (m.d. solution in 64.0% medium), sucrose (m.d. solution in 35.0% medium), sodium citrate (m.d. solution in 1.0% medium).

The resulting mixture of biomass and the cryoprotective medium was poured 6 ml each into sterile vials with a nominal volume of 10 ml, cooled at 4 to 6 °C.

To determine eutectic temperatures, we used the method proposed by L. Rey method, which consists in parallel measurement of temperature and electrical resistance of the substance during its freezing to minus 55 C and subsequent defrosting. In this case, the container with the studied substance was placed in a low-temperature freezing chamber HAIER DW 8 with a cascade refrigeration unit equipped with a compressor Secop (Danfoss). The electrodes were placed in parallel, with a gap of 2.5 mm between them. Registration of electric resistance values of the material was carried out by multimeter, temperature with the help of chrome thermocouples connected to the measuring regulator OVEN TRM 138-P.

When the liquid phase of the test substance is crystallized, its electrical resistance increases several times. The bend point on the graph will correspond to the temperature of complete freezing of the material when all moisture contained in it crystallizes.

During the subsequent defrosting process, the resistance will drop sharply. The first inflexion point on the graph corresponds to the upper eutectic boundary, the second to the lower boundary.

To confirm the results obtained, a synchronous thermal analyzer STA 449 F3 was used. The instrument's data acquisition and processing system are integrated into the main unit and connected to a PC.

4.2 Study of freezing of bacterial concentrates
To prepare the objects for further lyophilization and establishment of rational parameters, we investigated the process of freezing of bacterial concentrates with the cryoprotective medium. Samples were frozen to eutectic temperature using the following modes: 1 – the freezing rate 0.1 °C/min; 2 – freezing rate 0.45 °C/min.

When analyzing the freezing thermogram, it is possible to determine the cryoscopic temperature of the samples as well as the process duration.

4.3 Study of freeze-drying of bacterial concentrates
To determine rational parameters of the regime, we investigated the process of lyophilic drying of bacterial concentrates in the device LS 1000K «Prointekh» and through an experimental dryer with a combined system of heat and cold supply, consisting of a steam-compression refrigeration machine and thermoelectric, allowing heating the coolant due to the heat released on hot junctions of modules [9].

The main parameters of freeze-drying are the temperature of material sublimation depending on the degree of rarefaction in the chamber, final temperature, the density of heat flow of power supply sources, the temperature on the condenser (desublimation) surface.

For the study, the process in the device LS 1000K bacterial concentrate, pre-frozen in bottles was placed in a container with a flat bottom. During drying the following parameters were controlled: material temperature, condenser temperature, vacuum value, drying time.
For work out the model in the experimental device, the bacterial concentrate was placed in a specially designed multi-section unit with thermoelectric modules. During the drying process, in addition to the parameters mentioned earlier, the DC value was controlled and regulated.

At the end of lyophilization the appearance, residual moisture, the number of viable cells in the samples were determined. The number of colony-forming units (CFU/g) was determined by the cup method of sowing into plagiarized nutrient medium based on casein hydrolyzate.

5. Discussion of the results

Figure 1 and Table 1 shows the results of studies of eutectic temperatures of bacterial concentrates. Figure 1 shows the following eutectic points: $t_{le}$ – low eutectic point; $t_{ue}$ – upper eutectic point; $t_{cf}$ – complete freezing point.

The results obtained by the method of measuring electrical resistance depending on the temperature of the samples have good convergence with the data of the thermal analyzer. The error of values did not exceed 2.0-2.5%.

When crystallizing the liquid phase of the test substance, its electrical resistance increases several times. The kink point on the graph will correspond to the temperature of complete freezing of the material when all moisture contained in it crystallizes [10].

During the subsequent defrosting process, the resistance will drop sharply. The first inflexion point on the graph corresponds to the upper eutectic boundary, the second to the lower boundary. This fact can be explained by the fact that bacterial concentrates are biological materials containing various mineral and organic compounds. Each of these substances has a specific eutectic point, which leads to a eutectic zone of concentrates. Thus, the freezing temperature of bacterial concentrates should be several degrees below the upper eutectic point, i.e. in the range of minus 39 to minus 42 °C.

We have used the results obtained in the development of technological regimes for the pre-freezing process of materials subject to lyophilic drying.

![Figure 1. Temperature dependency of resistance of bacterial concentrate B. adolescentis: 1 – freezing; 2 – defrosting.](image)

Figure 2 shows the results of the freezing of bacterial concentrates study. Freezing process of bacterial concentrates with the cryoprotective medium was investigated for the preparation of objects for further lyophilization and establishment of rational parameters.
Table 1. Results of determining the eutectic temperatures of bacterial concentrates

| Indicator         | Temperature(°C) |
|-------------------|-----------------|
|                   | L. casei        | B. adolescentis |
| Lower border      | minus 32.4      | minus 33.6      |
| Upper border      | minus 37.8      | minus 38.1      |
| Complete freezing | minus 45.5      | minus 46.7      |

The whole freezing period is divided into three periods. The first one is characterized by material cooling to cryoscopic temperature with the subsequent supercooling. During the second temperature increases to cryoscopic one and remains constant for some time due to the latent heat of ice formation. The third one starts with further temperature decrease and ends when the value is below the eutectic point.

As a result, the cryoscopic temperatures of bacterial concentrates samples were determined: for lactic acid bacteria, *L. casei* the value was minus 1.4°C, for bifidobacteria *B. adolescentis* the value was minus 1.6°C. The results have a good agreement with the thermal analyser data. The error values did not exceed 1.5-2.0 %.

At a freezing rate of 0.1°C/min, the process duration for *B. adolescentis* was 190 min, for *L. casei* 176 min; at a freezing rate of 0.45 °C/min, the process duration for *B. adolescentis* was 96 min, for *L. casei* – 88 min.

Figure 2. Research of bacterial concentrate freezing:
1 – *B. adolescentis*, freezing speed 0.1 °C/min;
2 – *L. casei*, freezing speed 0.1 °C/min;
3 – *B. adolescentis*, freezing speed 0.45 °C/min;
2 – *L. casei*, freezing speed 0.45 °C/min.

Figure 3 shows time dependency of mass fraction of moisture for bacterial concentrate *B. adolescentis* and temperature of bacterial concentrate *B. adolescentis*.

The freeze-drying process can be divided into two periods. During the first period, moisture is sublimated from the material by energy supply. This period lasted over 400 min until the mass fraction
of moisture in the bacterial concentrate reached 10-12%. The second period (desorption) consists of drying the material to a mass fraction of 3.0-3.2 % moisture. During this period, it is necessary to reduce the heat flux density of heaters to avoid local overheating of the material.

During the lyophilic drying process, the following rational parameters were established: residual pressure in the chamber 0.05-0.06 mbar, the final temperature of the material 34-35°C, the temperature on the condenser (desorption) surface minus 55°C, the heat flux density of power supply sources in the first period 1.6-1.8 kWm⁻², in the second period 1.4-1.5 kWm⁻².

At the research of a drying mode in the experimental device with thermoelectric modules value of a direct current which has made 3.8-4.0A is established. Specific energy costs for drying in the experimental dryer decreased by 15% and amounted to 3.2 kW×h×kg.

Table 2 presents data on the determination of qualitative indicators of lyophilized bacterial concentrates.

The results indicate the achievement of high-quality indicators, which allows recommending the established modes for industrial use.

![Figure 3](image.jpg)

**Figure 3.** Study of the freeze-drying process of bacterial concentrates:
1 – time dependency of mass fraction of moisture for bacterial concentrate *B. adolescentis*;
2 – the temperature of bacterial concentrate *B. adolescentis*.

**Table 2.** Qualitative indicators of lyophilized bacterial concentrates.

| Indicator                          | Bacterial concentrate |
|-----------------------------------|-----------------------|
|                                   | *L. casei*             | *B. adolescentis* |
| Exterior view                     | pale yellow powder    | pale yellow powder |
| Mass fraction of moisture (%)     | 3.0                   | 3.2                |
| Vital cell count (CFU/g)          | 6.4×10¹⁰              | 8.5×10¹⁰           |
| Pathogenic microorganisms (in 10 g) | undetected           | undetected         |
| Squashing activity (h)            | 12                    | 12                 |

**6. Conclusion**

As a result of the complex definition of reasonable intervals of parameters of the process of lyophilization of bacterial concentrates, the given technological process is optimized. The optimization criteria are qualitative parameters of the product and energy consumption for freeze-drying. The
proposed parameters will reduce the duration of the process, which will increase the productivity of the equipment. Besides, a comparative analysis of the energy efficiency of the developed lyophilic dryer and existing analogues has been performed.

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