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RESEARCH ARTICLE **OPEN ACCESS**

OPTIMIZATION OF THE PROCESS OF LYOPHILIZATION OF MICROBIAL CULTURE OF BACILLUS SUBTILIS NBIMCC 2353 BY ULTRASOUND

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ABSTRACT

Lyophilization is a process in which water transforms from a solid to a gaseous aggregate state in a medium of vacuum and low temperature. In this type of processing of natural extracts, the biologically active substances in their composition remain unchanged. The continuous processing (24 - 72 hours), including freezing and drying in vacuum conditions and temperature regime from - 30°C to + 30°C, is associated with a high energy consumption, which is a disadvantage of the method. In the present publication, the authors present experimental data on the possibilities of using ultrasonic waves during lyophilization in order to reduce the process duration, production costs and to achieve low costs of the process. The subject of the research is a microbial culture, obtained from a producer strain of *Bacillus subtilis* NBIMCC 2353. It has been recorded that the use of an ultrasonic wave with a frequency of 40 kHz reduces the processing time by 32% which reduces the cost of the process, while maintaining high survival rates of the microbial cells. Mention the abstract for the article. An abstract is a brief summary of a research article, thesis, review, conference proceeding or any in-depth analysis of a particular subject or discipline, and is often used to help the reader quickly ascertain the paper's purpose. When used, an abstract always appears at the beginning of a manuscript, acting as the point-of-entry for any given scientific paper or patent application.

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INTRODUCTION

In recent years, freeze-drying (lyophilization) has emerged as a modern biotechnological decision in the production of pharmaceutical products and functional foods of high quality (Cheng et al., 2019; Siew, 2018). Lyophilization is a multiphase process that includes: freezing of the biomaterial to low temperatures; pre-drying, in which the water crystals sublimate when heated under vacuum and desorption of residual moisture (Berk, 2013). The obtained products have a fine structure and consistency, repeatedly lightened, with preserved nutrients, biologically active substances and taste and aroma complex. The residual moisture content is in the range - 2.5-5.0%, evenly distributed in the volume of the product and guarantees their long-term storage (Nacheva et al., 2014; Tsvetkov, 1979; Valchkov, Nacheva, 2013; Gallego-Juarez et al., 1999). However, in order to achieve these useful product characteristics, long processing and high costs are required. The drying time, that lasts from several hours to several days, limits the capacity of the industrial lyophilization.

Most often, heating plates or infrared heating are used as a process equipment that supply additional energy during the primary drying (ice sublimation under low pressure conditions) and accelerate the removal of water during the secondary drying (evaporation of the bound water to achieve the desired moisture content) (Morgan et al., 2006) by raising the temperature. Ratti (2001) reported that sublimation accounts for 45% of the energy consumption of lyophilization, while vacuum accounts for 26%, condensation 25% and freezing 4%. This necessitates the search for new possibilities to support sublimation by improving heat transfer in order to reduce the vacuum requirements by reducing the drying time (Schossler et al., 2012). Ultrasound is defined as sound waves with frequencies from 16 kHz to the MHz range. Sound waves are pressure waves that cause alternating areas of high and low local acoustic pressure. Ultrasound technology can be used to transmit mechanical energy in food systems and to accelerate mass transfer processes (Mulet et al., 2003). Therefore, it is a technology that complements the standard lyophilization process. By using ultrasonic vibration under vacuum conditions, ultrasonic waves can enhance the mobility of water molecules and facilitate the evaporation process. According to Zheng and Sun (2006), ultrasound has an effect on the size and distribution of crystals in the frozen product and prevents them from accumulating on the freezing surface. When used before or during freeze-drying, it can shorten the drying time and it has the potential to reduce the associated processing costs (Schossler et al., 2012). Patist & Bates (2008) report that low-power ultrasound with high-power and high-intensity in the range of 16–100 kHz, has been shown to be useful with a wide variety of applications in food processing, biopharmaceuticals and more. Ultrasound with high-power and low-frequency is a means for dehydrating food without affecting the main characteristics and quality of the product (Vladimir, 2009; Pakbin et al., 2014). In a research, Bantle, Eikevik and Grüttner (2010) reported an increase in the sublimation rate of 23.2% for ice and from 6% to 17.5% for various foods such as peas or cod with an atmospheric drying with the use of ultrasound.

Bacillus subtilis is one of the most widely used microorganisms in the biotechnology industry as a producer of various enzymes, vitamins and a wide range of biologically active substances needed for food production (fermented products, flavor enhancers, sweeteners, animal feed additives and more). *Bacillus subtilis* is a rod-shaped, gram-positive bacterium that occurs naturally in soil and vegetation. *Bacillus subtilis* develops in the mesophilic temperature range – 25-35°C. It has a strong adaptive capacity to stressful situations caused by acids, bases, ethyl alcohol and sudden changes in the temperature range (Martinez, 2013).

The purpose of the present research is to optimize the lyophilization process of a microbial culture containing *Bacillus subtilis* NBIMCC 2353 cells by incorporating an ultrasonic wave with a frequency of 40 kHz during the process, while maintaining a high number of viable cells.

MATERIALS, EQUIPMENT AND METHODS

MATERIALS

The producer strain *Bacillus subtilis* NBIMCC 2353, purchased from the National Bank for Industrial Microorganisms and Cell Cultures – Sofia (NBIMCC), was used for conduct of the experiment. The subject of the experiment is a microbial culture containing cells of *Bacillus subtilis* NBIMCC 2353 with an initial concentration of 1.4×10^8 CFU / ml.

Equipment

Lyophilization device *RAY-1A Atlas Danmark*, Incubator (*shaker BS/4*),UV/Vis spectrophotometer *Biochrom-Libra-S22*, Ultrasonic generator UCE-NT1000, hygrometer *Sartorius-thermo control YTC-01L*.

Methods – Microbiological

Method for culturing cell culture Bacillus subtilis NBIMCC 2353**:** The culture medium for the cultivation of the cell culture is composed of: 15 g/l Dextrose, 1 g/l FOS, 10 g/l Meet extract, 10 g/l Pepton, 5 g/l Yeast extract, 5 g/l NaCl, 1.3 g/l K_2HPO_3, 3 g/l $MgSO_4$ and distilled water. The culture medium is prepared in 2 two flasks of 1000 ml. It is autoclaved at a temperature of 121°C and 1.5 atm pressure for 25 minutes. The sterilized medium was cooled to a room temperature and inoculated under sterile conditions with *Bacillus subtilis* NBIMCC 2353. It is cultured in an incubator (*BS / 4 shaker*) at a temperature of 28°C, 100 rpm for 24 hours. After the completion of the cultivation process, the number of propagated cells in 1ml was determined spectrophotometrically.

Method for determining the number of cells: The *McFarland Standard* was used to determine the number of cells in cultural suspension using spectrophotometric measurement at wavelength $\lambda = 625$ nm of the sample and a comparison with standard solutions of $BaCl_2$ and H_2SO_4. After lyophilization, the ratio between the initial cultural suspension and the resulting lyophilisate is 10: 1. One gram of the lyophilisate, obtained from both samples, is rehydrated in 10 ml of sterile culture medium. The suspension obtained with this method was detected by the McFarland method at $\lambda = 625$ nm to determine the approximate number of cells.

Methods – Technological

Standard method for lyophilization: The cultural liquid in a volume of 1L is frozen at -30°C for 24 hours. The frozen sample was placed in a RAY-1A Atlas Danmark lyophilizer for vacuum drying that actively uses heating with programmed management of the temperature process. The temperature drying program, including a gradual increase in temperature from -30°C to + 30°C for a period of 48 hours, is started after reaching a vacuum of 1×10^{-1} mBar.

Modified method for lyophilization that uses a ultrasonic wave: The culture liquid in a volume of 1L is frozen at -30°C for 24 hours. The frozen sample was placed in a RAY-1A Atlas Danmark lyophilizer for vacuum drying. The temperature drying program, including a gradual increase in temperature from -30°C to + 30°C for a period of 48 hours, is started after reaching a vacuum of 1×10^{-1} mBar. To the power supply of the active heaters in the device, after the controller for temperature control, an ultrasonic generator UCE-NT1000 is included, set for operating frequency 40 kHz and radiated signal power 60 W, which leads to a distribution of 0.06 W / ml for irradiated 1L sample and emission time of 10 minutes (every time the active heating is switched on). Lyophilization was performed by both methods. Before the start of the process and at every 10 hours samples were taken to determine the degree of drying by measuring the residual moisture. The standard drying method lasts for 48 hours, and the modified method is performed until the establishment of a complete drying of the product.

Method for the estimation of the degree of drying: In order to determine the degree of drying, an aliquot amount of 1.5 g of the sample is taken from the temperature program every 10 hours to determine the presence of moisture by means of a *Sartorius-thermo control YTC-01L.*

Method for a comparative assessment of the cost of processing during lyophilization with RAY-1A Atlas Danmark: The lyophilizer *RAY-1A Atlas Danmark* has a capacity for processing products with a total volume of 10 L, distributed in 4 trays. The cost of electricity and water required for 1 hour of operation of the device is calculated for the cost of processing. The results obtained are presented for 1 L of working volume. In order to determine the cost of the process during the standard lyophilization method, the amount of electricity and water used for the full 24-hour drying cycle is measured. The obtained data are calculated for 1 working hour of the equipment and are displayed in a comparative table.

Statistical analyses

The analysis of each sample was performed in triplicate. Data are presented as mean ± standard deviation (SD) and were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

According to Fan et al. (2019) when treating a microbial culture of *Bacillus subtilis* with ultrasound at 80°C and 20 W/ml signal strength, the spores are inactivated. In order to maintain the survival of *Bacillus subtilis* NBIMCC 2353 cells in the used cultural medium, the maximum process temperature is reduced to + 30°C and the power of the ultrasonic signal - to 0.06 W/ml. The energy from the ultrasonic wave provides additional oscillation of the molecules in the microbial culture, leading to an increase in temperature with less energy consumption, compared to the use of an active heater. To determine the survival of *Bacillus subtilis* NBIMCC 2353 bacterial cells, a McFarland method of measurement was performed before and after lyophilization. The data are presented in Table 1.

Table 1. Bacterial cell survival after lyophilization (%)

Sample	Before lyophilization, CFU/ml	After lyophilization, CFU/ml	Survival, %
Standard method	0.4×10^{10}	3.2×10^{9}	80
Modified method	0.4×10^{10}	2.8×10^{9}	70

The results of the experimental trials show that when applying a standard method of lyophilization, the survival of the studied strain is high - 80%. When modifying the method with the inclusion of ultrasound, the survival rate is reduced by only 10%. The preservation of a high survival rate by freeze-drying with the help of ultrasound is due to the reduced drying temperature and the properly programmed lyophilization process. This proves the applicability of ultrasound as a support technique in the processes of freeze-drying of a microbial culture containing cells of *Bacillus subtilis* NBIMCC 2353. During the lyophilization process, the degree of drying was determined by measuring the residual moisture of the experimental variants. Figure 1 shows the results of the measurements of the samples taken at the beginning, at the end and in every 10 hours during the drying process.

It was established that when using ultrasonic waves, at the 30th hour from the initiation of the process, a low residual humidity of the microbial culture is achieved - 2.63% and lyophilization can be terminated. With the standard method, the process lasts until the 40th hour when the desired values for residual humidity of the product are achieved. The basis of the experiment is the hypothesis that ultrasonic waves accelerate the rate of sublimation and shorten the process time. Water sublimation is perfmormed in an open system with active heating with energy supplied from the outside and operating in vacuum conditions. With active heating, energy is transferred by means of thermal radiation in the infrared region (λ = 0.74 - 2.5 µm; λ = 2.5 - 50 µm; λ = 50 - 2000 µm) with a frequency range from 300 GHz to 430 THz. In order to optimize the process and to reduce the cost of the maintenance of the system, another type of energy transmitted through a mechanical ultrasonic wave, with a frequency of 40 kHz, is included. In this combination, the impact of both types of energy (thermal radiation in the IR area and mechanical wave with a frequency of 40 kHz) on the water molecule achieves a significant acceleration of the rate of rupture of the weak hydrogen bonds, which causes a rapid transition from a solid to a gaseous state. To illustrate the process of acceleration in the system, we express the change to increase the rate of rupture of weak hydrogen bonds by changing the rate of sublimation of water.

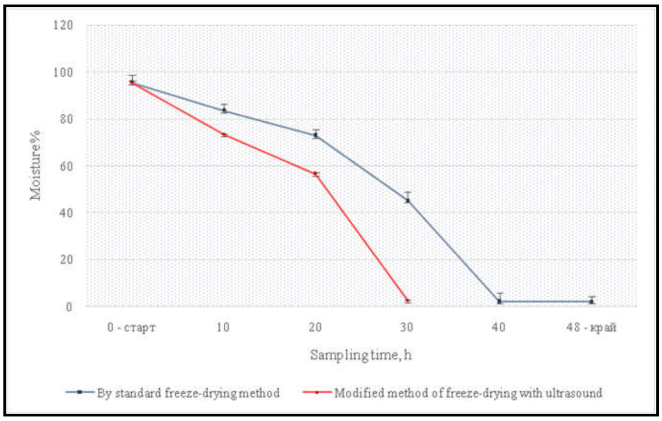

Figure 1. Residual moisture after lyophilization

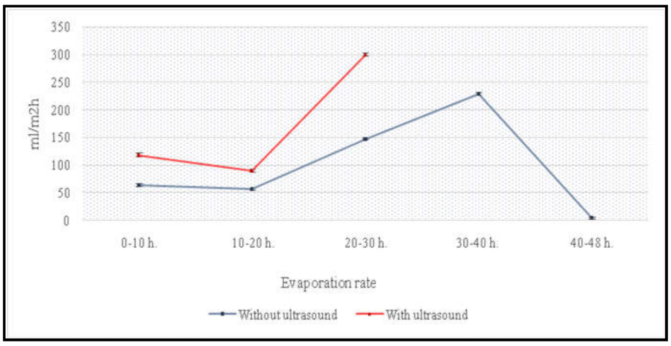

Figure 2. Rate of water sublimation by both methods

Figure 2 presents the results of the measurement of the rate of water sublimation during the individual stages of the experiment. The data are obtained on the basis of the amount of water passed into the gaseous state and evaporated from a surface of 1 m^2 for 1 hour. In order to evaluate the achieved efficiency with lyophilization of the microbial culture, the direct costs for the processing with lyophilizer RAY-1A Atlas Danmark are checked.

Table 2 presents the results of the analysis of the direct costs required to carry out the lyophilization drying for one cycle with and without the use of ultrasonic wave for the same amount of dried material. The obtained results prove the effectiveness of the application of an ultrasonic wave to optimize the total direct costs for lyophilization of a microbial culture containing cells of *Bacillus subtilis* NBIMCC 2353.

Table 2. Costs necessary to carry out the process

Cost	Unit Price, BGN	Without ultrasound quantity, (per cycle)	Without ultrasound value, BGN (per cycle)	With ultrasound quantity (per cycle)	With ultrasound value, (per cycle)	Change in consumption,%
Electric energy, per kW / h	0.2284	576	131.55	360	82.22	37.5
Water, for (L)	0.0028	312	0.87	195	0.546	37.24
Total direct costs	-	-	132.42	-	82.77	37.49

Conclusion

The applicability of ultrasonic waves in the lyophilization of a microbial culture containing cells *Bacillus subtilis* NBIMCC 2353 has been proved. The optimization of freeze-drying by incorporating ultrasound into the technological process leads to the preservation of a high survival rate of microbial cells.

Experimental data show that ultrasonic waves change the rate of sublimation, which leads to a reduction in the duration of the process. This contributes to the reduction of energy consumption and therefore the costs of production of lyophilized products from *Bacillus subtilis.*

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