STUDY OF PHYSICOCHEMICAL AND THERMAL PROPERTIES OF L-PHENYLALANINE AMMONIA-LYASE

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Abstract: Physicochemical and thermal properties of L-phenylalanine ammonia-lyase preparation were studied. Thermal gravimetric analysis of physical and chemical phenomena occurring in the enzyme upon heating was conducted. Heating curves were registered. Kinetic parameters of the evaporation process were identified. Stability of the enzyme to freezing was verified. Cryoscopic temperature of a concentrated L-phenylalanine ammonia-lyase sample was identified. Chemical (dehydration, dissociation) and physical transformations accompanied by exothermic and endothermic effects were found to occur during freezing. Results of the thermal gravimetric analysis demonstrated feasibility of freeze-drying of L-phenylalanine ammonia-lyase preparation and allowed for determination of the optimum temperature ranges for thermal treatment and prediction of operational parameters of the drying process.

Key words: L-phenylalanine ammonia-lyase, physicochemical and thermal properties, heating curves, cryoscopic temperature, lyophilization

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INTRODUCTION

Despite the many years of production of dry biological preparations, no simple, cost-efficient, and precise enough method to calculate parameters of freeze-drying process at each stage has been proposed so far [1, 2]. Above all, this is due to the inherent complexity of the drying process, its physical and mathematical modeling, and complexity of mathematical tools to solve the differential equations describing thermodynamics of lyophilization process, especially of such complex systems as biopolymers [3, 4].

Therefore, studies aimed at determination of operating conditions for lyophilization of an enzyme preparation, L-phenylalanine ammonia-lyase, and development of freeze-drying technology are of topical issue [5].

L-phenylalanine ammonia-lyase catalyzes the reaction of reverse deamination of L-phenylalanine to *trans*-cinnamic acid and ammonia.

The enzyme is of interest as a therapeutic agent for phenylketonuria treatment and may be used for both direct therapy of phenylketonuria and production of food products free of phenylalanine [4, 5]. Besides the medical applications, the enzyme may be used in biotechnology for L-phenylalanine production from *trans*-cinnamic acid [6, 7].

The enzyme is subjected to freeze-drying to preserve its activity during storage.

Also, L-phenylalanine ammonia-lyase may be stored in glycerol solutions.

To determine operating conditions for freeze-drying of any product, including an enzyme preparation, thermophysical characteristics, together with physicochemical properties, should be known. Thermophysical characteristics are necessary for determination of both rational operating conditions and technological parameters [6, 7].

Therefore, the aim of the present work was to study physicochemical and thermophysical characteristics of an enzyme preparation of L-phenylalanine ammonia-lyase that would allow for selection of optimal freeze-drying modes.

MATERIALS AND METHODS

L-Phenylalanine ammonia-lyase preparation was the subject of the study.

To study L-phenylalanine ammonia-lyase as a subject of drying, thermal gravimetric analysis of physicochemical events occurring in the enzyme upon heating was to be performed.

Changes in weight of sample were registered in function of temperature upon thermal gravimetric analysis of L-phenylalanine ammonia-lyase preparation.

Study of thermophysical characteristics of the enzyme preparation was performed by the first buffer method of two temperature–time intervals [7].

Then, enzyme stability to freezing was verified. Aliquot of purified protein was diluted in 0.1 M Tris-HCl buffer, pH 8.5. Protein concentration in the sample was 0.2 mg/mL. Part of it was used to determine activity and concentration of the protein. The remaining sample was placed in a freezer at –18°C overnight. After thawing on ice, activity was determined [8, 9].

For further experiments on lyophilization, protein preparation after secondary purification was used. Sulfate suspension was centrifuged and the sediment was dissolved in 50 mM Tris-HCl, pH 8.5. Then, it was dialyzed against the same buffer (with two buffer exchanges) during 20 h. No loss of activity occurred upon dialysis. Dialyzed preparation had specific activity of 2.99 U/mg protein. Protein concentration was 8.51 mg/mL. Equal volumes of protein (255 μL each) were placed in three 0.5-mL glass vials. Twenty five microliter 5% D-trehalose solution was introduced into one of the vials, 25 μL 5% polyvinylpyrrolidone solution, into another one, and 25 μL Tris-HCl buffer, pH 8.5, into the third one. Activity of the preparation after dialysis was used as control value. Vials were covered with several layers of cotton tissue and left at –70°C overnight [10].

For thermophysical studies, weighted amount of the enzyme (200 mg) was placed in a platinum cup with a cap 9.5×10^{-3} m in diameter; sensitivity of the weighing part was 50 mg, heating rate, 5°C/min, DTG = 1/10, and DTA = 1/10; studies were performed at atmospheric pressure.

At the next stage, L-phenylalanine ammonia-lyase was placed in a cup of electronic balance set in a vacuum box. Energy was supplied with an infrared source. Temperature of the product was measured with a KSP-4 potentiometer and maintained within the range of 22–27°C under residual pressure of 1.6–2 kPa. Thus, weight of the sample in the process of concentrating was determined. After the vacuum box, samples were placed in a desiccator, where they were kept for 4–6 h to balance humidity over the sample volume. Water content in the sample was determined by drying according to State Standards.

Then, cryoscopic temperature of the concentrated sample was determined.

RESULTS AND DISCUSSION

Cryoscopic temperature is the difference between the freezing point of bidistilled water and freezing point of a product determined by cryoscopy and expressed in temperature measurement units.

Thermophysical characteristics of L-phenylalanine ammonia-lyase before and after freezing

Physical state	Thermal diffusivity $a \cdot 10^7$, m^2/s (± 5%)	Thermal conductivity λ, W/(m·K) (± 5%)	Density ρ, kg/m^3 (± 2%)	Specific heat per unit mass c_m, J/(kg·K) (± 5%)
liquid preparation (t = 18°C)	1.37	0.56	1013	4166
frozen preparation (t = –24°C)	11.97	2.18	920	1979
dry preparation (t = 20°C)	14.94	3.36	1192	1888

Cryoscopic temperature is a necessary parameter in the development of processes of low-temperature treatment of products and materials. Therefore, information on cryoscopic temperature is of practical importance. Thermophysical characteristics of L-phenylalanine ammonia-lyase before and after freezing are presented in the table.

Protein concentration influences the cryoscopic temperature of enzymes. Besides, since considerable amount of yeast extract and sodium chloride is present in the enzyme preparation, cryoscopic temperature in function of these two components' concentration was studied.

The results were compared with cryoscopic temperatures of sodium chloride aqueous solution (Fig. 1).

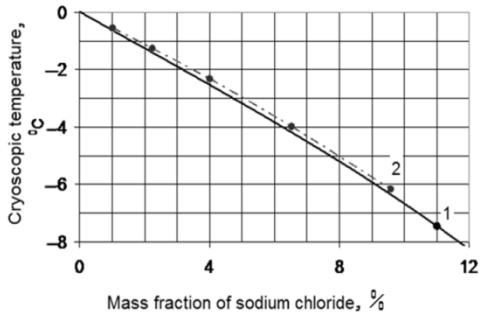

Fig. 1. Cryoscopic temperature of solution in function of sodium chloride concentration: 1, sodium chloride aqueous solution; 2, enzyme preparation.

The curves presented in Fig. 1 confirm that the manner of crystallization of the enzyme preparation is overall the same as that of sodium chloride aqueous solution. However, cryoscopic temperatures of the enzyme preparation were somewhat higher (by 0.1–0.2°C) than the relevant sodium chloride solution. Apparently, this is due to hydrophobic properties of the protein in the enzyme preparation. Nevertheless, the influence is not significant, therefore, aqueous solution of sodium chloride may be used as a model to study freezing of L-phenylalanine ammonia-lyase enzyme preparation.

As mentioned above, cryoscopic temperature of enzymes is affected by protein concentration. Therefore, cryoscopic temperature in function of protein concentration was studied (Fig. 2).

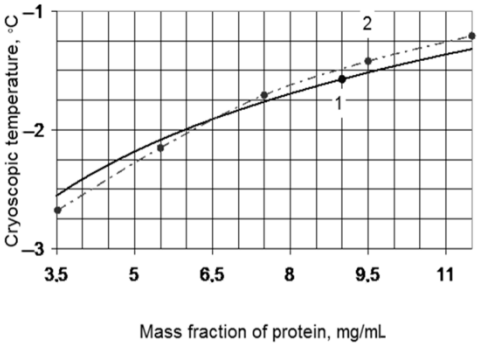

Fig. 2. Cryoscopic temperature of L-phenylalanine ammonia-lyase enzyme preparation in function of protein concentration.

The results evidenced that there is a reverse logarithmic dependence between the cryoscopic temperature

of the enzyme and protein concentration in the range of 3.5 to 11.5 mg/mL.

Increase in protein concentration in the preparation from 3.5 to 7.5 mg/mL resulted in increase of its cryoscopic temperature by 0.3–0.6°C; further increase from 7.5 to 11.5 mg/mL resulted in the change in cryoscopic temperature of 0.6–1.3°C. Change in cryoscopic temperature of the enzyme preparation in function of protein concentration was probably due to transfer of free water to bound water.

In calculations of specific enthalpies of the enzyme preparation, enthalpy of the preparation corresponding to the temperature of –40°C was considered zero enthalpy.

Calculation of thermal conductivity coefficients was performed by additivity method. In general, it is not applicable to thermal conductivity calculations, however, it provides for sufficient reliability of calculated data for food products since thermal conductivity coefficients of constituents are values of the same orders of magnitude (except for gases), and the products are isotropic.

The model of maximum coefficient of efficient thermal conductivity of a compound is represented by alternating parallel plates of components under heat flowing in direction parallel to the plates.

Weight fraction of frozen water (Fig. 3) was calculated in temperature range of –24–0°C.

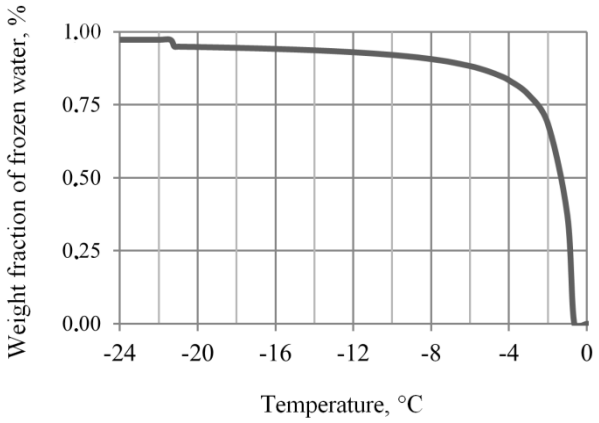

Fig. 3. Weight fraction of frozen water in L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

Specific thermal capacity (Fig. 4), enthalpy (Fig. 5), coefficients of thermal conductivity (Fig. 6) and thermal diffusivity (Fig. 7), as well as density (Fig. 8) values, were determined for the L-phenylalanine ammonia-lyase enzyme preparation in function of temperature in the range from –40 to 25°C.

To evaluate applicability of the elaborated techniques, we calculated the values characterizing thermophysical properties of the L-phenylalanine ammonia-lyase enzyme preparation in liquid and frozen state and the calculation results were compared with experimental ones.

Comparison of the values characterizing thermophysical properties of the L-phenylalanine ammonia-lyase enzyme preparation determined experimentally with those calculated, in general, evidences applicability of the proposed model for determination of the thermophysical characteristics. Errors of thermophysical characteristics determination by calculation, if compared to the experimental data, are 4–5.5%, for thermal diffusivity, 3–5.5%, for thermal conductivity, and 1–3%, for specific heat per unit mass values. The technique may be used to determine thermophysical characteristics of liquid, frozen, and dry preparation, which is necessary for modeling of the processes of L-phenylalanine ammonia-lyase freeze-drying.

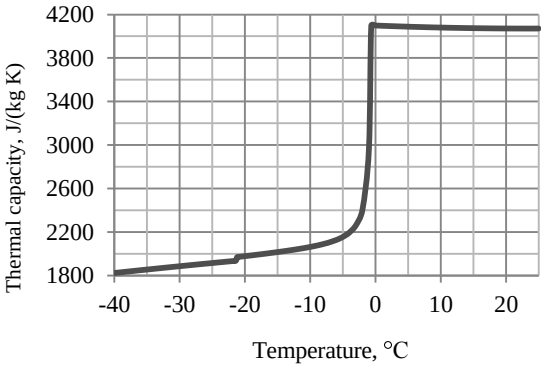

Fig. 4. Specific thermal capacity of L-phenylalanine-ammonia lyase enzyme preparation in function of temperature.

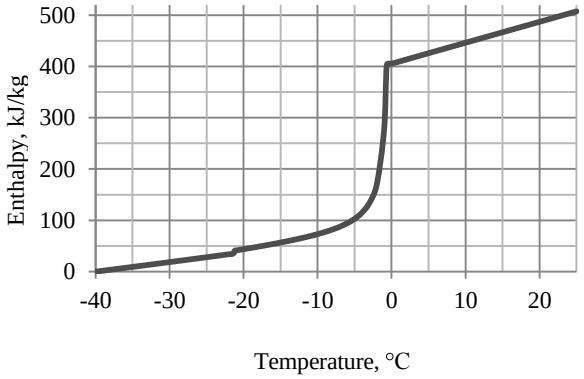

Fig. 5. Specific enthalpy of L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

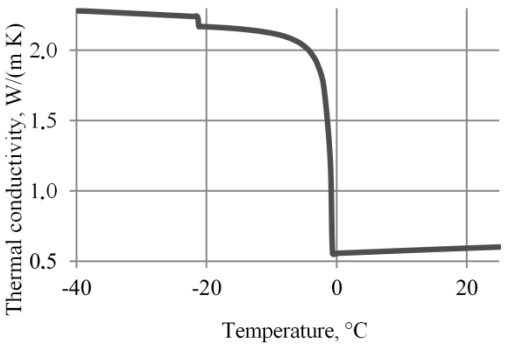

Fig. 6. Coefficient of thermal conductivity of L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

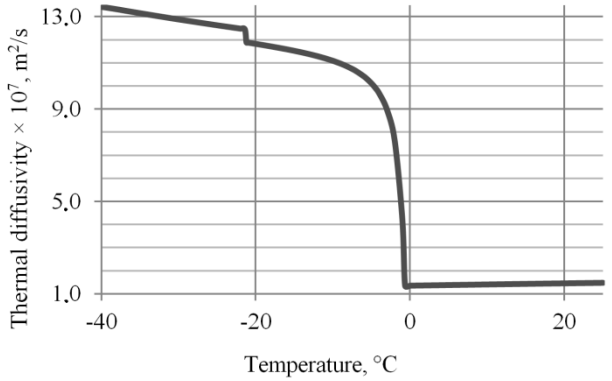

Fig. 7. Coefficient of thermal diffusivity of L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

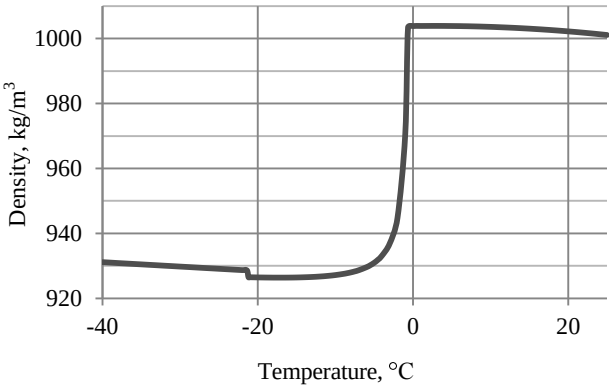

Fig. 8. Density of the L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

The amount of water removed from the product upon drying depends on the structure and physicochemical properties of the dried subject. The effect of the level of temperature stabilization of the dried layer on the duration of lyophilization was studied. Typical drying curves of the L-phenylalanine ammonia-lyase enzyme preparation under the studied levels of stabilization of dry layer are presented in Fig. 9.

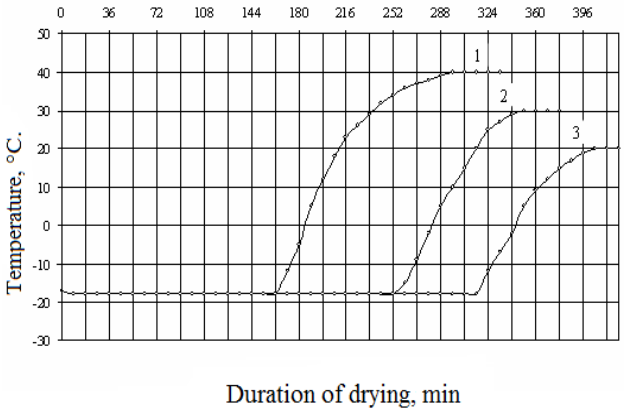

Fig. 9. Typical drying curves of the L-phenylalanine-ammonia lyase preparation under the studied levels of stabilization of dry level: 1, 40°C; 2, 30°C; and 3, 20°C.

Increase in the level of dry layer stabilization resulted in the increased amount of water removed during sublimation and decreased amount of water removed during heating. Temperature of 20–40°C is the most favorable for the process of L-phenylalanine ammonia-lyase enzyme preparation drying since in this range of temperatures water removal is intensified and drying duration is shorter.

In the study, microstructure of the dry preparation was investigated. Figure 10 demonstrates microstructure of L-phenylalanine ammonia-lyase enzyme preparation obtained upon freeze-drying at different temperatures of stabilization on surface of the dry layer.

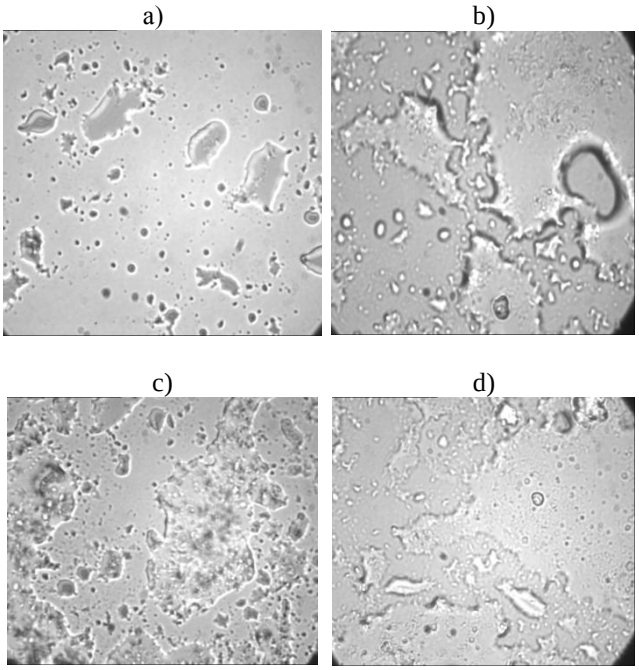

Fig. 10. Microstructure of dried enzyme preparation (magnification of 500×) at different temperatures of stabilization on surface of the dry layer: (a) 15°C; (b) 20°C; (c) 30°C; and (d) 40°C.

As follows from Figure 10, dried preparation of L-phenylalanine ammonia-lyase obtained by freeze-drying contains approximately the same particles with hard and smooth surface.

During sublimation process under industrial conditions, data on specific energy consumption per unit water removed from enzyme preparation in function of temperature and duration of concentrating process is of considerable interest. Figure 11 demonstrates dependence of specific heat consumption during sublimation drying of L-phenylalanine ammonia-lyase enzyme preparation.

As follows from the presented data, increase in temperature of stabilization of the dry layer results in increase in specific heat consumption during sublimation drying of the enzyme preparation.

Specific heat consumption in vacuum-dryers is known to be in the range of 0.8–1.2 kW/kg per 1 kg removed water. According to the data of our studies, specific heat consumption in the process of

L-phenylalanine ammonia-lyase drying at 20–40°C is 0.7–0.9 kW/kg removed water. Utilization of freeze-dryer allows for reduction of heat expenses on the enzyme preparation drying, if compared to vacuum-dryers, by 10–20%.

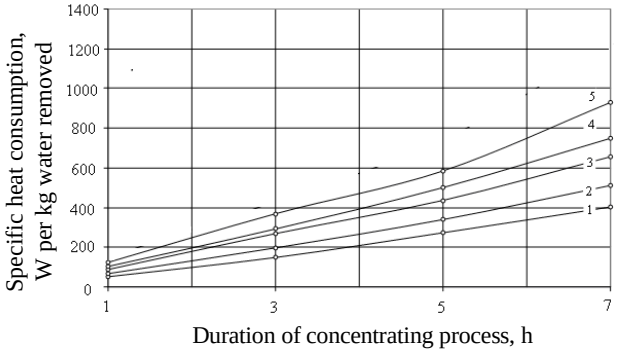

Fig. 11. Specific heat consumption during sublimation process of L-phenylalanine ammonia-lyase enzyme preparation in function of stabilization temperature of the dry layer: 1, 5°C; 2, 15°C; 3, 20°C; 4, 30°C; and 5, 40°C.

At temperatures of dry layer stabilization of 20–40°C, weight fraction of residual water does not exceed 5.0%. At temperatures below 20°C, water content in dried enzyme preparations is higher (6.0 to 10.5%).

Duration of sublimation drying of L-phenylalanine ammonia-lyase enzyme preparation was estimated at different temperatures of drying, that is, 20, 30, and 40°C (Fig. 12).

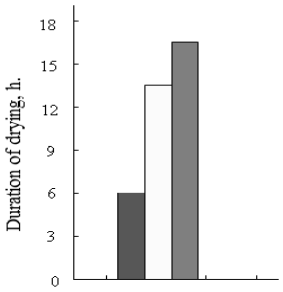

Fig. 12. Duration of sublimation drying of L-phenylalanine ammonia-lyase enzyme preparation in function of drying temperature:
- drying temperature 20ºC;
- drying temperature 30ºC;
- drying temperature 40ºC.

Results presented in Figure 12 evidence that increase in the temperature of enzyme drying from 20 to 40 ºC results in increase of drying duration by 10 h. Therefore, 6 h of the enzyme drying at 20ºC was chosen as optimal duration.

Density of heat flow (heat load) is as important a parameter of the process as is temperature. Density of heat flow is the amount of heat supplied from heaters to a unit area of the product being dried (kW/m^2).

The rate at which rational temperature of lyophilization is reached depends on the heat load value. At low values of heat load, time to reach rational (required) temperature of drying of biological subject grows, which results in the increase in total duration of the drying process. High heat loads may lead to worsening of quality and defects in the dried product. Worsening of quality is manifested through formation of overdried layers on top of the product.

Overdried layers affect considerably the process of drying and quality of dried products. Overdried layers have lower coefficient of thermal conductivity, which, on one hand, increases the gradient of temperatures between the surface and the bulk subject; on the other hand, overdried layers prevent mass exchange between the subject and the environment. The water evaporated inside a product needs to overcome additional resistance of overdried layers. Therefore, heat load value is to be considered in the process of development of rational drying modes.

Rational heat load should take into account temperature of drying, physicochemical parameters of product being dried, duration of drying, and energy consumption.

Studies on the selection of rational heat load for the L-phenylalanine ammonia-lyase enzyme preparation were performed at the following values: 9.2; 8.28; 7.36; 6.44; 5.52; 4.6; 3.68; 2.76; 1.84; and 0.92 kW/m^2.

Figures 13–15 demonstrate the curves of temperature and heat load upon drying of the L-phenylalanine ammonia-lyase enzyme preparation. Upon increase in the heat load, the rate at which preparation surface reached the temperature of 20ºC increased. At heat load of 9.2 kW/m^2, target temperature of the surface of preparation was reached within 55 min; at 5.52 kW/m^2, within 60–65 min; and at 1.84 kW/m^2, within 140–150 min.

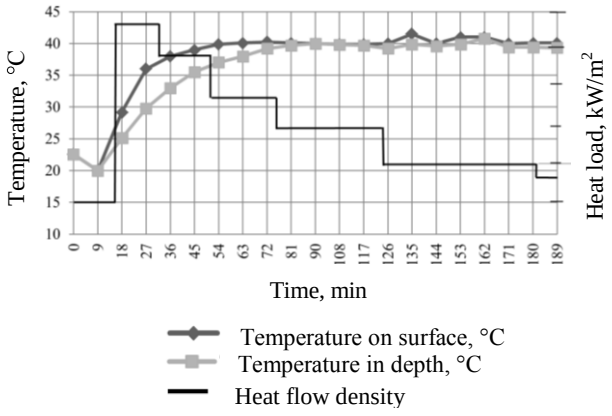

Fig. 13. Sublimation drying curves of the L-phenylalanine ammonia-lyase enzyme preparation at heat load of 9.2 kW/m^2.

Equalizing of temperature over the volume of the enzyme preparation at high values of heat load occurs faster. Temperature in the thickness of the preparation equalized to the temperature of the surface at heat load values of 9.2, 5.52, and 1.84 kW/m2 within 80, 110, and 215 min, respectively.

Lyophilization is a complex technological process, on which parameters of the final product depend.

Freeze-drying regime is considered rational if it allows for preservation of physicochemical properties of raw material at the minimal energy consumption. For rationalization of the technological process of sublimation drying of the L-phenylalanine ammonia-lyase preparation, effect of residual lyophilization pressure on product characteristics was studied.

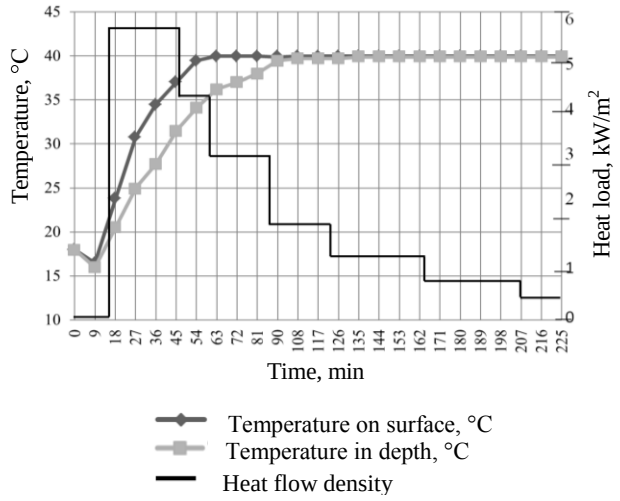

Fig. 14. Sublimation drying curves of the L-phenylalanine ammonia-lyase enzyme preparation at heat load of 5.52 kW/m^2.

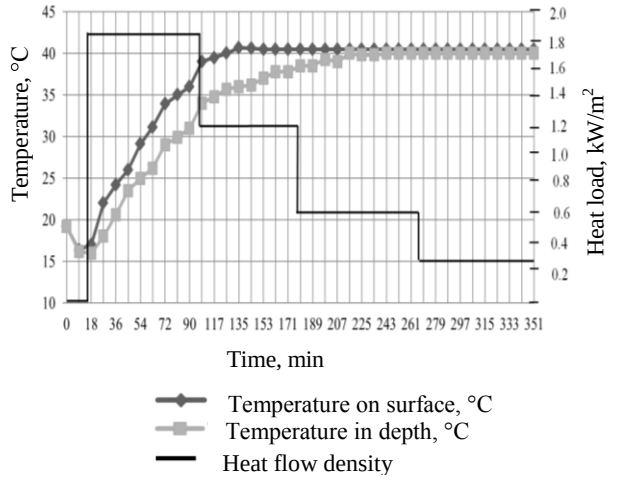

Fig. 15. Sublimation drying curves of the L-phenylalanine ammonia-lyase enzyme preparation at heat load of 1.84 kW/m^2.

The process of lyophilization was studied at residual pressure values of 5–6, 10–11, and 14–15 Pa. These values of lyophilization residual pressure were chosen basing on the literature data and required parameters of freeze-drying during rationalization of the parameters.

Temperature of water vapor saturation at residual pressure values of 5–6, 10–11, and 14–15 Pa was –48–(–46), –42–(–40), and –39–(–38)°C, respectively.

Duration of freeze-drying process increases with increase in the residual pressure. Increase in the residual pressure from 5–6 to 10–11 Pa results in the increase of process duration by 60–80 min; increase from 5–6 to 14–15 Pa results in the increase by 120–160 min. Basing on the experimental data, empirical function of L-phenylalanine ammonia-lyase freeze-drying duration dependence on residual pressure in the range of 5–6 to 14–15 Pa was obtained.

Under the residual pressure of 5–6 Pa, heat consumption increases due to increase in the coefficients of working time of the freezer and vacuum pumps up to 0.85–0.9, if compared to the residual pressure of 10–11 Pa, when the value is 0.6–0.7.

Increase in specific heat consumption at 14–15 Pa does not depend upon the coefficient of working time of the freezer and vacuum pumps and is equal to 0.6–0.7, similar to the residual pressure of 5–6 Pa. Increase in specific heat consumption at 14–15 Pa occurs due to increase in the duration of lyophilization process.

Therefore, optimal parameters of lyophilization process of the L-phenylalanine ammonia-lyase enzyme preparation are the following: duration of the process, 6 h; heat flow density, 9.2 kW/m^2, and residual pressure, 6 Pa.

Typically, temporal changes in average values of moisture and temperature over the volume of material being dried are termed kinetics of the drying process.

Drying process is most accurately described by drying curves (drying rate vs. material humidity) and temperature curves (material temperature vs. material humidity). Work of dryers of different efficiency cannot be compared by changes in weight of material in the process of drying. For this purpose, graphical images of humidity evolution in the material, or drying curves, are used.

Data to build the curves are typically obtained in laboratory by registering a sample weight and temperature in the process of drying. Drying is typically performed by warm air. For freeze-drying, constant parameters of a regime are material temperature and residual pressure value. Naturally, transfer of the laboratory study data to industrial conditions (where drying is typically performed under alternating mode) requires corrections.

Change in the volume-averaged humidity of material with time is graphically depicted by a curve termed drying curve. In general, drying curve contains several parts corresponding to different stages of drying.

The amount of water frozen out is another important characteristics of the product that is of practical importance in choice of sublimation temperature and determination of optimal technological modes of drying. The data obtained are used in thermal technics calculations.

In the process of water freezing, concentration of solutes in liquid phase grows since only water molecules and a few solutes turn to solid phase, especially at low temperatures. This process may be modeled by increase in concentration of dry substances under vacuum concentrating [10].

Therefore, it was found that sublimation drying of L-phenylalanine ammonia-lyase enzyme preparation proceeds in two stages: constant-rate and decelerating drying. The enzyme drying curves in terms of heat load vs. time, temperature vs. time, and moisture fraction vs. time were obtained and investigated. Drying rate curves

were plotted using graphic differentiation. Values of maximum drying rate of the preparation in function of layer thickness were determined. The amount of water due to polymolecular adsorption was analyzed and found to be 4–9%.

Mean value of bound water was $\sigma = 0.223$ kg/kg.

Therefore, thermal gravimetric analysis demonstrated-feasibility of L-phenylalanine ammonia-lyase dehydration by freeze-drying; results of the analysis allow for determination of optimal temperature ranges for thermal treatment and forecast technological parameters of the drying process.

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