ISSN 2310-9599 (Online)

КемТИПП

FOODS AND RAW MATERIALS

Vol.1 (№ 2) 2013

The Ministry of Education and Science of the Russian Federation

Kemerovo Institute of Food Science and Technology

Foods and Raw Materials

Vol.1, (No. 2), 2013

ISSN 2308-4057 (Print)
ISSN 2310-9599 (Online)

Published twice a year.

Founder:
Kemerovo Institute of Food Science and Technology (KemIFST),
The juridical address:
bul'v. Stroitelei 47, Kemerovo, 650056 Russia

Editorial Office, Publishing Office:
650056, Russia, Kemerovo, bul'v. Stroitelei 47, office 1212,
tel.: +7(3842)39-68-45
http: frm-kemtipp.ru
e-mail: fjournal@mail.ru

Printing Office:
650056, Russia, Kemerovo,
ul. Institutskaya 7, office 2006,
tel.: +7(3842)39-09-81

Signet for publishing
December 23, 2013
Date of publishing
December 27, 2013
Circulation 300 ex.
Open price.

ISSN 2308-4057 (Print)
ISSN 2310-9599 (Online)

The Edition is registered by Federal Service for Supervision in the Sphere of Telecom, Information Technologies and Mass Communications (Media Registration Certificate PI no. FS77-52352 dated December 28, 2012).

The Edition « Foods and Raw Materials» is included in the Russian index of scientific citation (RISC) and registered in the Scientific electronic library eLIBRARY.RU
The information about edition is published in international reference system of periodical and proceeding editions of "Ulrich's Periodicals Directory".

Kemerovo Institute of Food Science and Technology (KemIFST), bul'v. Stroitelei 47, Kemerovo, 650056 Russia

CONTENTS

FOOD PRODUCTION TECHNOLOGY

ANALYSIS OF LIVING AND REPRODUCTIVE PARAMETERS OF MICROORGANISMS

A. A. Maiorov

Siberian Research Institute for Cheese-Making,
Russian Academy of Agricultural Sciences,
ul. Sovetskoi Armii 66, Barnaul, 656016 Russia,
phone: (3852) 56-46-12, e-mail: sibniis.altai@mail.ru

Received August 18, 2013; accepted in revised form September 3, 2013

Abstract: A probability correlation between various transitions and the number of microorganisms at different stages of growth has been analyzed. Comparison of the given parameters with those of the environment (temperature, active acidity, oxidation-reduction potential, etc.) allows defining the influence of each parameter. The obtained results and correlations can be recommended for modeling the growth of microorganisms in different environments, cheese mass being one of them.

Key words: microorganism growth, environment, cheese, cultivation process, optimization algorithm

UDC 637.1
DOI 10.12737/2045

The ability of microorganisms to grow plays an important role in dairy production [1, 2]. The microorganisms, owing to enzymes they produce, impact the texture, smell, and flavor of a dairy product. The probiotic characteristics of such a product play an important role, too. To ensure successful reproduction of microorganisms, appropriate growth conditions must be provided.

Reproductive capacity is best assessed by using the probability theory. In this case, the probability of division of one cell living in specific conditions, characterized by the presence and concentration of a substrate, water activity, active acidity, the salt weight fraction, and a number of other parameters that influence the cell's life, is calculated [3, 5, 7].

This can be done on the basis of either special or previously conducted experiments provided that the conditions of such experiments were recorded. Both methods require compiling a rather large database that helps predict the behavior of bacteria in any given conditions. As complicated as it may seem at first glance, this task requires a strictly formalized approach to the description of the properties of both microorganisms and their environment. The present-day methods of mathematical modeling make it possible to predict the behavior of objects and their interaction with the environment [6, 10].

As regards the growth of microorganisms, a distinction should be made between a closed (uncontrolled) and controlled environment. Partially controlled systems can also exist. An uncontrolled system is such that is not exposed to external influences or when such exposure is negligible. The ideal uncontrolled system is a thermally insulated and hermetically sealed tub containing a substrate with the original number of microorganisms. Nominally, cheese mass at the ripening stage can be considered such a system [11, 13]. The main physical and chemical processes in cheese are influenced by ferments, i.e., chemical components that make up the cheese mass. Microorganisms are actively involved in this process as they take up nutrients, release metabolic products, and change the environment. Their activity during cheese ripening can only be affected by changing the temperature. A decrease in the temperature results in the reduced reproductive rate; an increase in the temperature accelerates the rate of cell division.

The majority of cheeses ripen within a temperature range of 8–20^{o}C. During cheese ripening, its moisture content changes owing to water evaporation off the surface. This content is not large as opposed to the total cheese mass, but it can be of paramount importance as it influences the life of microorganisms.

Therefore, cheese can be referred to a group of systems with partially controlled parameters. In practical terms, it means that the living conditions of microorganisms inside cheese mass can only be controlled by changing its ripening and storage temperature.

The manufacture of fermented milk products is controlled more easily. Fermented milk products are normally manufactured in tanks equipped with a temperature control system (cooling and heating) and agitators. This setup makes it possible to stir the mass during production and influence the temperature. Moreover, various ingredients that influence the living conditions of microorganisms can be added to the mixture. Such ingredients may be salt, sugar, flavoring agents, preservatives, emulsifiers, stabilizers, etc. This system, although isolated from external influences, can be controlled in a wider context. However, the volume of this system and, consequently, its resources are limited, which means that only a certain number of microorganisms can be

grown in this volume. Their maximum concentration is limited not only by the nutrients in the substrate but also by a variety of other factors.

A so-called flow-through fermenter that ensures control over the living conditions of microorganisms (bacteria) is used to produce various biopreparations. In addition to agitators and temperature control tools, such machines are also fitted with a waste products discharge system, a nutrient supply system, and a system that regulates the gas-phase composition supplied to the substrate. This fermenter must be equipped with special tools to control the parameters of cultivation of microorganisms. The main output controlled parameter can be either the volumetric number of microorganisms (biomass volume) or the concentration of waste products produced by microorganisms (ferment). These two indicators do not always correlate with each other. In this event, it is important to have information on how the qualitative and quantitative parameters of the substrate (environment) affect the output parameters (the number of microorganisms and the concentration of the ferment of interest). This information is obtained through special tests by varying environmental parameters and measuring the efficiency of separate and cumulative influence of the environmental parameters. On the basis of the obtained regularities, a cultivation control program is formed to determine the main and supplementary algorithms of cultivation optimization [14, 15].

By analyzing the capabilities of various systems, it is possible to determine ranges of their controllability and build a control algorithm focused on the optimization of the output parameter.

In practical terms, there is a necessity to analyze the dynamics of bacterial flora growth in a given environment. With a high microorganism concentration in a volume unit, the population influence on the chemical composition of the environment is very significant and often plays a decisive role. Special chemostats that ensure steady cultivation conditions can be used for quite an accurate study of the influence of environmental parameters on the growth of microorganisms [12, 13].

When cultivating in a changing environment, it is more difficult to analyze the effect of individual factors, which leads to the ambiguous interpretation of the obtained results. A more detailed picture of the growth of microorganisms in an environment can be obtained using the living environment reconstruction method (LER). Analysis of the dynamics of microflora growth in cheeses is an example of the application of this method.

The growth of microflora in cheese is assessed by the results analyzed at different production stages. As cheese transitions from one stage to the next, it is very difficult to take into account the influence of various factors on both the cheese and its microflora. In reality, as each factor is a time-dependent variable, it is a challenging task to measure a share of influence that each of them exerts on the microflora growth.

Additional information relating to the influence of such factors can be obtained on the basis of the dynamics of changes in the microflora population. For this purpose, time sampling of the microflora growth at given intervals must be conducted. The sampling interval must be proportionate to the period of microorganism generation, for instance, 0.5 h. A differential curve can then be built, which, in its simplest form, is a difference in the microflora population at the previous and the next sampling interval:

$$D = Qi+1 – Qi.$$

Ideally, each cell of microorganisms is divided in two:

$$Qi+1 = 2Qi;$$

i.e., the population of microorganisms doubles at every interval.

In practical terms, not all microorganisms are capable of division.

The division capability is determined by a combination of factors and can be defined as follows:

$$Ki = \frac{Qi+1}{2Qi},$$

where Qi+1 is the number of microorganisms in the next generation; Qi is the number of microorganisms in the previous generation; and Ki is a coefficient that characterizes what portion of microorganisms achieves their capacity to divide.

This coefficient can be interpreted as a cell division probability at interval i. This helps calculate the probability of cell division at every division stage. In this case, it is more accurate to speak not about the cell division probability but about a cell division coefficient at a given stage, which is a cumulative influence coefficient embracing all factors affecting the microorganisms.

When the general influence regularities of each factor on the probability of MO cell division are known, it is possible to determine the share of influence of each factor at various stages.

When analyzing a population change as an elementary process of cell division, the approach based on the assessment of division probability becomes appropriate. As a matter of fact, the reproduction of microorganisms is based on the division of individual microorganisms, and the population growth, on the whole, depends on what portions of the microorganisms will divide. In other words, the division process can be thought of as random or stochastic. A cell transition from being undivided into being divided (two cells) is a discrete process. The probability of division, in this case, is a function of a whole number of factors, a time factor being one of many.

In some cases, this factor can be of paramount importance since normally the microflora growth is described in “number”–“time” coordinates.

The use of random processes to describe microorganism growth allows moving on to criterial assessments, which are very important when studying regularities based on the multistage influence of many factors.

When using deterministic functions, any indicator can be calculated with a 100% certainty by changing its functionally dependent argument; this, however, cannot be applied to cell division. Even when dealing with strictly defined parameters of reproduction environment

and a strictly selected strain of microorganisms, it cannot be stated with assurance that a cell will divide into two cells at a strictly determined interval (for instance, 23.4 minutes). This only means that a cell division process can occur within 22–25 minutes under specific cultivation conditions. In other words, there is a high probability that a cell will divide between the 22nd and 25th minutes of cultivation. In terms of the strict wording, one should speak of the probability of cell division within a given timeframe. A cell division probability curve can be asymmetrical due to the different nature of restrictions which accelerate or decelerate the division process. Variations in the cultivation conditions change both the coordinates of the curve maximum point and the steepness of the ascending and descending slopes. When the cultivation conditions move outside of an optimal zone, the probability decreases; as the distance grows, the probability value becomes more negligible. The envelope of these curves represents a biokinetic zone, i.e., a zone where the existence of microorganisms with a specified probability is possible.

The cell division probability describes an increment or, rather, an increment rate over time. To complete the picture, it is necessary to consider the duration of a cell reproduction age, which can be quite lengthy but not lead to the increase in cell population. Finally, an important element in the overall picture of the growth of microorganisms is the end of their life or the cell death.

Depending on the environmental conditions, microorganisms can stay at each 'stage of life' for a different period. On the whole, life cycle duration for a microorganism can be assessed on the basis of the probabilities for such microorganisms to stay in three main states.

In mathematical terms, it is not quite appropriate to use the system of differential equations to describe the reproduction of microorganisms as it can be used only for continuous functions, whereas the division process in itself, as it has been previously stated, is discrete, i.e. discontinuous.

Speaking about the application of mathematical systems, it is worth noting that the queuing theory along with the Markov chains is an effective method of analyzing the reproduction of microorganisms. [17]

Thus, transition from one state to another can be described by calculating the probability or intensity of transitions. The reproduction process can be represented as a transition from one state to another.

All microorganisms (cells) can be conventionally divided into three groups representing different states. The first group includes microorganisms that are capable of dividing within the timeframe of one generation (the productive category).

The second group includes microorganisms that are presently nonproductive but have the potential to divide at the next stages (the reversible category). This category can be further divided into subcategories depending on their previous history. This category must include cells in an adaptation state after division, cells exposed to mutation or antagonistic pressure from other cells, or cells deprived of sufficient nutrition, etc. These factors can be specified when modeling biochemical and biophysical processes. In any case, it is assumed that cells that belong to this category maintain the potential for future division.

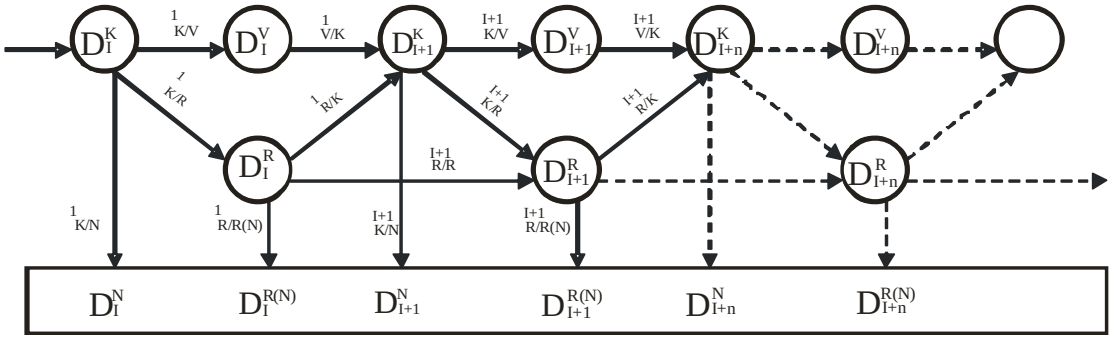


Fig. 1. Pattern of microorganism division.

The third group comprises microorganisms whose reproductive capability is irreversibly lost (the irreversible group). This group cannot be identified by microbiological tests such as inoculation of media but can distort the interpretation of the dynamics of growth of microorganisms when the population is measured by nephelometric or turbidimetric analysis. This subtle detail of using population data should be taken into consideration as it plays an important role in building an accurate model and interpreting test results. Regarding lactate microflora, it is assumed that cells formed as a result of mother-cell division are equal. Accumulation of defects leading to infertility of cells happens with an equal degree of probability for both branches that evolve in the reproduction process. It does not mean a limitless number of cell divisions even when reproduction conditions are favorable. Part of a cell population can be exposed to mutation as a result of errors accumulated during successive generations, and, consequently, take on new properties; the other part loses the capability to reproduce owing to irreversible changes in the genetic apparatus.

When building a mathematical model of population development based on the Markov chains, an overall schematic diagram can be represented by a marked graph that includes all states of the system with the specified transition intensities (Fig. 1). The number of states depends on the complexity of the process model under consideration.

At the initial stage, there is a population containing D_1 of microorganisms. Influenced by a combination of internal and external factors, the population transits into state *V* with a number of productive units equal to D_1^V, thus, distinguishing *N* (irreversible) and *R* (reversible) categories with populations D_1^N and D_1^R, respectively. The transition intensity from state *K* into states *V*, *N*, and *R* is described by coefficients $\lambda^1_{K/V}(\tau)$, $\lambda^1_{K/N}(\tau)$, and $\lambda^1_{K/R}(\tau)$. The value of these coefficients depends on the combination of factors that influence the population. When there exists a probability of reverse transitions, appropriate coefficients $\lambda_{n/i}$ can be used to describe the processes. Each transition may be characterized by certain intensities. In this case, based on the given definitions, part of the reversible category can replenish the productive category in the next reproduction period; the remaining part of the reversible category may transit into the similar category during the future reproduction process.

In terms of the formal approach, the part of the reversible category that transits into the similar category of the next period can be considered as a part of the irreversible category as it plays the same role in population development as the irreversible group under the steady process of changing environmental parameters. However, for building an accurate model and for the correct interpretation of its behavior during research, the transition structure should be kept in the state as it is shown in Fig. 1.

The pattern shown in Fig. 1 can be replaced with a recursive pattern, i.e., repeating itself at every stage of reproduction. Then, as was mentioned before, the reproduction pattern will have five groups of microorganisms (five states). In reality, there are three groups involved in the pattern: productive, reversible nonproductive, and irreversible nonproductive (dying cells). The fourth and fifth groups are made of a hypothetical part of microorganisms consisting of microorganisms in a metastable state and a group that represents a new generation, i.e., a productive group from the previous generation doubled in number.

The state graph for such system is shown in Fig. 2. A group of microorganisms in state S_1 (the metastable state) transits into states S_2 (the reversible group) and S_5 (the irreversible group). Part of microorganisms transits from state S_1 into state S_3 (the productive group). The intensity of transitions from one state to another is characterized by appropriate coefficients λ_i.

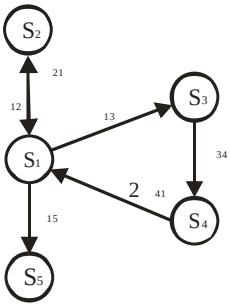


Fig. 2. State graph of the system of microorganisms.

Based on the assumption that the number of microorganisms found in an environment in any state of system S_i is a random value with an exponential distribution function and transition intensity at this stage (λ_i), the reproduction process corresponding to the flow graph can be described by a system of equations:

$$P_1(\tau) = (\lambda_{21} - \lambda_{12} - \lambda_{13} + 2\lambda_{41} - \lambda_{15})P_1(\tau);$$
$$P_2(\tau) = (\lambda_{12} - \lambda_{21})P_1(\tau);$$
$$P_3(\tau) = \lambda_{13}P_1(\tau) - \lambda_{34}P_3(\tau);$$
$$P_4(\tau) = \lambda_{34}P_3(\tau) - 2\lambda_{41}P_4(\tau);$$
$$P_5(\tau) = \lambda_{15}P_5(\tau),$$

where $P_i(\tau)$ is probability that a microorganism at time τ is in state S_i.

By analyzing the correlation between various transition probabilities and the number of microorganisms at different stages of growth and by measuring these parameters against those of the environment (temperature, active acidity, oxidation-reduction potential, etc.), it is possible to determine the degree of influence that each of them exerts. The obtained results and correlations can be used in further modeling the growth of microorganisms in different environments, cheese mass being one of them.

All probabilities can become permanent provided the cultivation conditions remain invariable.

Trial experiments in modeling the growth of microorganisms in a closed uncontrolled environment with a limited supply of nutrients, have proved that a suitable model created on the basis of the approach suggested in this article, is quite possible.

REFERENCES

1. Tutel'yan, V.A., *Novye tekhnologii v nauke o pitanii* (New Technologies in Food Science), Moscow, 2001.
2. Lipatov, N.N., Aspects of modeling amino-acid balance in food products, *Pishchevaya i pererabatyvayushchaya promyshlennost' (Food and Food Processing Industry),* 1986, no. 4, pp. 49–52.
3. Maiorov, A.A., Razrabotka metodov upravleniya biosistemoi syra s tsel'yu sovershenstvovaniya traditsionnykh i sozdaniya novykh tekhnologii (Development of methods of cheese biosystem regulation to improve conventional and create new technologies), *Extended Abstract of Doctoral Dissertation in Engineering*, (Kemerovo, 1999).
4. Osintsev, A.M., Braginskii, V.I., Ostroumov, L.A., and Gromov, E.S., Ispol'zovanie metodov dinamicheskoi reologii dlya issledovaniya protsessa koagulyatsii moloka (Application of dynamic rheology in studying milk coagulation process), *Khranenie i pererabotka sel'khozsyr'ya (Agricultural Commodities Storage and Processing),* 2002, no. 9, pp. 46–49.

5. Shamanova, T.P., Mikrobiologicheskie i tekhnologicheskie podkhody k proizvodstvu fermentirovannykh produktov (Microbiological and technological approaches to fermented milk production), *Molochnaya promyshlennost' (Dairy Industry)*, 1998, no. 3, pp. 18–20.
6. Adler, Yu.P., Markova, E.V., and Grankovskii Yu.V. *Planirovanie eksperimenta pri poiske optimal'nykh uslovii (Planning an Experiment in Search of Optimal Conditions),* Moscow, Mir, 1976.
7. Polishchuk, P.K., Derbinova, E.S., and Kazantseva, N.N., *Mikrobiologiya moloka I molochnykh produktov (Microbiology of Milk and Dairy Products),* Moscow, Pishchevaya promyshlennost', 1978.
8. Babak, O., Ispol'zovanie mikroorganizmov v pishchevoi promyshlennosti (Usage of microorganisms in the food industry, *Produkt BY (Product BY)*, 2008, no. 11. http://www.produkt.by
9. Borunova, S.B., and Furik, N.N., Analiz vyzhivaemosti termofil'nykh molochnokislykh mikroorganizmov pri zamorazhivanii (Analysis of survival capabilities of thermophilic lactate microorganisms during freezing) in *Sovremennye tekhnologii sel'skokhozyaistvennogo proizvodstva: materially XIV Mezhdunar. nauch.-prakt. konf. (Present-day Technologies in Agricultural Production: Proceedings of the XIV International Research and Practice Conference)*, Grodno, 2011, part 2, pp. 273–274.
10. Glazova, A.A., Botina, S.G., and Tipiseva, I.A., Ustoichivost' otechestvennykh proizvodstvennykh kul'tur bakterii roda *Lactobacillus* k antibakterial'nym preparatam (Resistance of nationally manufactured *Lactobacillus* bacteria cultures to antimicrobials), in *Sbornik trudov Moskovskogo gosudarstvennogo universiteta pishchevykh proizvodstv (Collected Works of Moscow State University of Food Industries),* Moscow, 2010, pp. 67–77.
11. Gudkov, A.V., *Syrodelie: tekhnologicheskie, biologicheskie i fiziko-khimicheskie aspekty: monografiya (Cheese-Making: Technological, Biological, Physical, and Chemical Aspects: A Monograph),* Moscow, DeLi Print, 2003.
12. Emel'yanov, S.A., Khramtsov, A.G., Suyunchev, O.A., et. al., Vliyanie temperatury na razvitie mikroorganizmov v moloke i molochnykh produktakh (Temperature impact on microorganism growth in milk and dairy products), *Vestnik SevKavGTU (North Caucasus STU Bulletin)*, 2006, no. 2, pp. 54–57.
13. Knyazhev, V.A. and Tutel'yan, V.A., Mikroorganizmy i pishcha. Risk i pol'za (Microorganisms and food: Risks and benefits), *Vestnik Rossiiskoi akademii meditsinskikh nauk (Herald of the Russian Academy of Medical Sciences)*, 2000, no. 12, pp. 3–6.
14. Holt, G., Krieg, N., Sneath, P., Staley, J., and Williams, S., *Bergey's Manual of Determinative Bacteriology*, in 2 vols., 9th ed., Baltimore, Williams & Wilkins, 1994; Moscow, Mir, 1997.
15. Ostroumov, L.A., Prosekov, A.Y., Kurbanova, M.G., and Kozlova, O.V., Pitatel'nye sredy dlya bifidobakterii (Nutritional environments for bifidus bacteria), *Molochnaya promyshlennost' (Diary Industry)*, 2010, no. 1, pp. 20–21.
16. Prosekov, A.Yu., Kozlov, S.G., and Kurbanova, M.G., Vliyanie fermentatsii zakvasochnoi floroi na nekotorye svoistva molochnogo belkovo-uglevodnogo syr'ya (Influence of starter culture fermentation on certain properties of dairy protein and carbohydrate raw materials), *Khranenie i pererabotka sel'khozsyr'ya (Agricultural Commodities Storage and Processing)*, 2004, no. 9, pp. 31–33.
17. Gorban, A.N., Gorban, P.A., and Judge, G., Entropy: The Markov Ordering Approach, *Entropy,* 2010, vol. 12, no. 5, pp. 1145–1193.

EFFECT OF FREEZING ON THE BIOCHEMICAL AND ENZYMATIC ACTIVITY OF LACTOBACILLUS BULGARICUS

E.V. Korotkaya and I.A. Korotkiy

Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone/fax: +7 (3842) 73-43-44, e-mail: krot69@mail.ru

Received March 1, 2013; accepted in revised form April 1, 2013

Abstract: The problem of preserving the viability, stability and activity of thermophilic lactic acid bacteria Lactobacillus bulgaricus upon freezing is considered. The effect of different freezing conditions and low-temperature storage on the biochemical and morphological properties and stability of the DNA of L. delbrueckii ssp. bulgaricus has been investigated. Sensory evaluation has been carried out for non-frozen bacterial starter cultures containing L. bulgaricus, and their basic physical and chemical parameters (titratable and active acidity and relative viscosity) have been determined. The influence of low temperature on these parameters has been investigated. The effect of freezing and low-temperature storage on the antagonistic activity of L. delbrueckii ssp. bulgaricus strains has been elucidated. The optimum freezing and storage temperatures for the starters containing L. bulgaricus have been determined.

Key words: freezing, low temperature storage, biochemical and morphological properties of L. bulgaricus, antagonistic activity

UDC 573.4.086.13
DOI 10.12737/2046

INTRODUCTION

The quality of cultured dairy foods depends directly on their production technology and on proper selection, preservation, and subsequent culturing of the starter microflora. The microorganism conservation methods known today consist in bringing the vegetative cells of the microorganisms into an anabiotic state. Since these cells are incapable of passing to the endogenous dormancy state, immersing them into exogenous dormancy (by drying, lyophilization, freezing, etc.) and bringing them out of this state produce stressful situations that cause death of a considerable part of the microorganism population and lead to phenotypic and genotypic changes. Bacterial cells are known to induce nuclease in response to a cold shock, so the lethal effect of low temperatures is due to DNA destruction [1, 2].

Advantages of freezing technologies and low-temperature storage of bacterial starter cultures over the other conservation methods are that they are simple and convenient, require only a small amount of preparative work, and allow rapid recovery of the stored material from the frozen state. Compared to drying and lyophilization, freezing causes less damage to microorganism cultures and leaves them more viable [1, 3–5]. In addition, freezing rarely induces genetic changes [1, 3, 4, 6].

The purpose of this work was to study the effects of various freezing temperatures and conditions and low-temperature storage conditions on the biochemical properties, enzymatic activity, morphology, and genetic stability of thermophilic lactic acid microorganisms of the *L. bulgaricus* genus.

EXPERIMENTAL

Bacterial starters were obtained from lyophilized bacterial starter cultures produced by Barnaul'skaya Biofabrika Co. (*L. Bulgaricus*; BBV = Bulgarian bacillus, viscous; BBNV = Bulgarian bacillus, nonviscous) and from the *L. bulgaricus* strains B-3964, B-6516, B-3141, B-6543, and B-6515 from the Russian National Collection of Industrial Microorganisms (RNCIM) at the Institute of Genetics and Selection of Industrial Microorganisms.

The lactic acid bacteria culturing medium was reconstituted nonfat dry milk (RF State Standard GOST R 52090-2003), which had no off-flavors or foreign odors and did not contain inhibitors.

The milk was sterilized in an autoclave (steam sterilizer, DGM-500 model) for 10–15 min at a pressure of 0.1 MPa and a temperature of 121 ± 2°C.

The laboratory fermentation starter was prepared under sterile conditions in an abacterial air environment PCR box (Laminar-S). Lyophilized starter cultures were introduced into sterile milk cooled to 38–39°C, which was then thoroughly stirred. Fermentation was performed in a TSO-1/80 SPU thermostat at 40–41°C until the formation of a clot of desired quality.

The starter cultures were frozen at –45, –25, or –10°C in air and in a liquid coolant (ethanol). Freezing was carried out in special-purpose low-temperature chambers.

The starter culture temperature during freezing was measured with chromel–copel thermocouples, whose signal was received by an MVA-8 analog input module

and an AS-4 interface transformer and was recorded on a personal computer.

The DNA of lactic acid bacteria was isolated using a bacterial genomic DNA isolation kit (Sintol, Moscow) [7]. The 16S rRNA gene was amplified on a Tertsik amplifier (DNK-Tekhnologiya, Moscow) using thermostable Taq polymerase (SibEnzim, Novosibirsk) according to the manufacturer's recommendations. The following species-specific primers were used in amplification: 16SbulF: 5'- CAA CAG AAT CGC ATG ATT CAA GTT TG (26) and 16SbulR: 5'- ACC GGA AAG TCC CCA ACA CCT A (22) [7].

The antagonistic activity of *L. bulgaricus* was determined by perpendicular-stroke coculturing [8] on the surface of dense nutrient medium no. 2. Experiments were performed on 16- to 16-h-old bacterial cultures grown in liquid nutrient medium no. 1.

Liquid nutrient medium no. 1 had the following composition: skim milk hydrolysate (amine nitrogen, 200–250 mg%), 250.0 mL; concentrated yeast autolysate (amine nitrogen, 200–250 mg%), 100.0 mL; agar, 0.8 g; distilled water, to 1 L; 20% NaOH solution, to pH 6.4 ± 0.1. Dense nutrient medium no. 2 had the following composition: skim milk hydrolysate (amine nitrogen, 200–250 mg%), 250.0 mL; concentrated yeast autolysate (amine nitrogen, 200–250 mg%), 100.0 mL; agar, 20.0 g; distilled water, to 1 L; 20% NaOH solution, to pH 6.4 ± 0.1.

The morphological properties of thermophilic lactic acid bacterial cultures were studied by immersion microscopy using a Rathenow microscope with lens 90. The specimens were stained by Gram's method or with methylene blue.

The following physical and chemical properties of the laboratory fermentation starters were determined: titratable and active acidity (pH), relative viscosity at 25°C (measured with Ostwald's capillary viscometer), number of viable lactic acid microorganisms (measured by the limiting dilution analysis), and pathogenic microflora content (quantified according to the USSR State Standard GOST 9225-84).

RESULTS AND DISCUSSION

The most important properties characterizing the industrial applicability of a starter culture are its acid production capacity and fermentation activity, the structural and mechanical properties of its clot, the micropattern and organoleptic properties of the resulting clot, and viable microflora content.

The bacterial starter cultures obtained in this study were characterized by a white, delicate, and uniform clot with slight signs of whey separation. The fermented milk clots were readily dividable and acquired a uniform texture upon stirring. All of the starters had a pleasant odor, a fermented milk flavor, and no off-flavors or off-odors. The color of the clot was milk white and was uniform throughout the product bulk. There was no pathogenic microflora in the laboratory starters. The lactic acid microorganism content and some physical and chemical properties of the fresh bacterial starter cultures are listed in Table 1.

Table 1. Characteristics of the fresh bacterial starters

Bacterial culture	Lactic acid microorganism content, CFU/cm^3	pH	Relative viscosity
BBV	$6.0 \cdot 10^9$	4.2	4.31
BBNV	$3.0 \cdot 10^9$	4.2	1.75
B-3964	$4.5 \cdot 10^9$	4.2	1.72
B-6516	$5.0 \cdot 10^9$	4.0	1.80

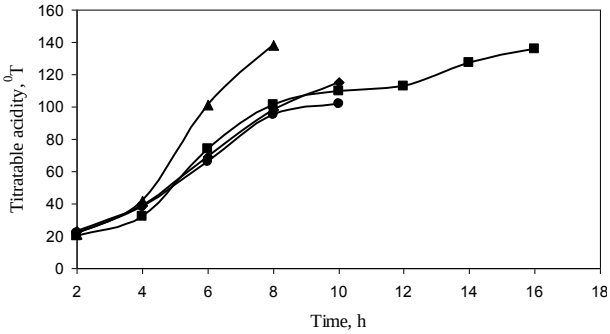


Fig. 1. Titratable acidity as a function of fermentation time: ■, BBNV; ▲, BBV; ♦, B-6516; ●, B-3964.

The variation of the titratable acidity of milk during clotting is illustrated in Fig. 1.

All of the bacterial starters had the necessary acidity. The relative viscosity of the bacterial starter obtained using BBV was 2.6 times higher than that of the starters from the nonviscous starter cultures.

The next step of our study was investigation of the effect of low temperature on the viability of the starter microorganisms.

The freezing resistance of microorganisms depends on several factors, including the microorganisms' genus and species, the stage of their development, temperature, freezing rate, freezing medium, and storage time. The effect of low temperature on microorganisms is characterized in terms of intracellular and extracellular changes. The heaviest damage is caused by intracellular ice formation, which disrupts plasma membranes and cell walls. In addition, ice formation increases the concentration of intracellular and extracellular solutions, and this leads to protein denaturation and to the disruption of permeability barriers [2].

The fresh starters were poured into 10-mL test-tubes under sterile conditions and were then frozen at –45, –25, or –10°C in air or in the liquid coolant. After freezing, the tubes with fermentation starters were stored in heat-insulated containers at the temperature equal to the freezing temperature. The frozen starters were stored for 6 months.

The frozen starters were examined to determine their microbiological, biochemical, and physicochemical characteristics. Before being examined, the starters frozen at –10°C were thawed in a refrigeration chamber at 4–8°C. The starters frozen at –25 or –45°C were thawed in a water bath at 20°C.

The data characterizing the dependence of lactic acid microorganism content of the laboratory starters on the

freezing technique and storage time are presented in Table 2.

Table 2. Number of *L. bulgaricus* cells in the starters after freezing

Storage time, days	Number of microorganisms, CFU/cm^3 · 10^{-9}					
	t = –10°C		t = –25°		t = –45°C	
	1*	2**	1	2	1	2
BBV						
1	2.19	3.00	4.50	4.60	5.21	6.00
14	1.10	2.10	3.30	3.80	4.00	4.50
30	0.50	1.30	2.50	3.00	3.50	3.60
60	0.08	0.45	1.50	2.00	2.20	2.50
90	0.03	0.12	0.60	1.10	1.50	1.70
180	0.009	0.009	0.30	0.50	0.5	1.10
BBNV						
1	1.10	1.20	1.90	2.00	2.50	2.70
14	0.30	0.50	1.40	1.50	2.00	2.20
30	0.10	0.25	1.10	1.20	1.60	2.00
60	0.01	0.07	0.60	0.70	1.1	1.40
90	0.007	0.007	0.30	0.45	0.80	1.10
180	0.003	0.005	0.12	0.20	0.30	0.50

* Freezing in air; ** freezing in the liquid coolant.

The freezing temperature and rate and the storage temperature can significantly affect the survival of the microorganisms [9–11]. Throughout the storage period, the highest survival rate of the microorganisms was observed in the starters frozen and stored in the liquid coolant at –45°C, i.e., at the minimum storage temperature used in this study. This finding is in agreement with data of other authors [9–11]. After 6-month-long storage, the average survival rate of the microorganisms was 17% of their initial number; that is, the microorganism content decreased by less than one order of magnitude. The survival rate of the microorganisms in the starters frozen in air at –45°C was somewhat lower: in 6 months: their number was approximately 10 times smaller than their initial number. The number of microorganisms in the starters frozen at –25°C was, on the average, 5–10% of their initial number, depending on the freezing regime. The lowest survival rate was observed for the microorganisms in the starters frozen at –10°C.

The rather high survival rate of the microorganisms frozen in the liquid coolant can be explained as follows. The efficiency of heat transfer in the freezing of the starters in the liquid coolant is much higher than in their freezing in air. As a consequence, the freezing time for the starters frozen in the liquid coolant is much shorter than for the starters frozen in air. Accordingly, the water crystallization rate for the starters in the liquid coolant is one order of magnitude higher. These freezing conditions minimize the destructing factors associated with water crystallization in the cells and intercellular space, which cause death of a large number of microorganisms in the starter.

The high survival rate of the lactic acid microorganisms after freezing does not ensure that they completely retain their properties and viability. The functional activity of the starter bacteria was judged from acid formation intensity data.

The acid formation activity was determined from the time required to reach pH 4.5 in culturing the microorganisms in reconstituted dry skim milk. Table 3 lists acid production activity data for the lactic acid bacterial cultures frozen in the liquid coolant at –45°C.

Table 3. Acid production activity data for *L. bulgaricus* bacteria

Bacterial starter	Fermentation time for 100 mL milk + 1 mL starter, h	
	before freezing	after freezing
BBV	7–8	7–8
BBNV	15–16	16–17
B-3964	10–11	10–12
B-6516	10–11	10–12

These data demonstrate that, after 6-month-long storage, the starters retained their high biochemical activity; the fermentation time increased, on the average, by 1 h. The growth dynamics of the thawed *L. bulgaricus* cultures that were frozen in the liquid coolant at –45°C and were stored for 6 months is illustrated in Fug. 2.

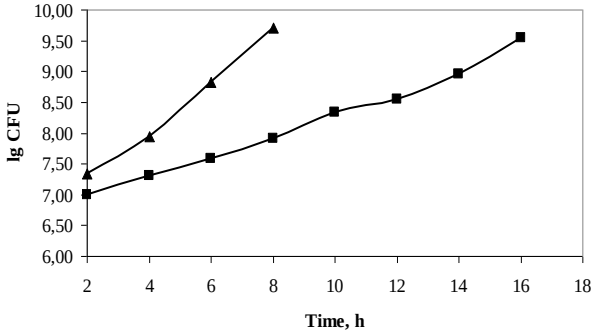


Fig. 2. Growth dynamics of *L. bulgaricus* cells in milk fermentation with the (■) BBNV and (▲) BBV starter cultures.

An important physicochemical property of a starter culture is its viscosity. Fig. 3 shows how the relative viscosity of BBV starter cultures as a result of freezing and storage under different conditions.

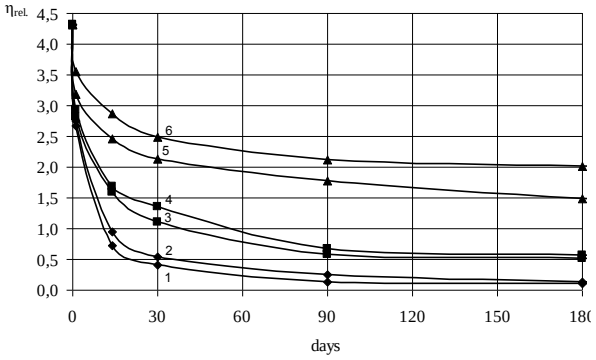


Fig. 3. Effect of freezing under different conditions on the relative viscosity of the BBV starter culture. Freezing in air: (1) –10°C, (3) –25°C, and (5) –45°C. Freezing in the liquid coolant: (2) –10°C, (4) –25°C, and (6) –45°C.

These data suggest that the strongest viscosity-reducing effect is exerted by freezing as such: the relative viscosity decreases by a factor of 1.2–2.4, depend-

ing on the kind of starter culture and freezing technique. The largest, 2.4-fold decrease in relative viscosity was observed for BBNV frozen in air at –10 C; the smallest decrease in relative viscosity, for BBV frozen in the liquid coolant at –45°C. The decrease in relative viscosity of the bacterial starter cultures at –10°C is nearly independent of whether they were frozen in air or in the liquid coolant. In 14 days, the relative viscosity of the bacterial cultures frozen in air decreased, on the average, by a factor of 4.6 and that of the cultures frozen in the liquid coolant decreased by a factor of 3.7.

The marked decrease in the relative viscosity of the bacterial starter cultures frozen in air or in the liquid coolant at –10°C is due to the fact that slow freezing yields large ice crystals outside the cells, thus changing the initial ratio of the volumes of the intercellular and intracellular spaces through water redistribution and water-to-ice transition.

In extracellular ice formation, the growth of ice crystals in the intercellular space reduces the cell size. This leads to cell compression and to the formation of folds in the cell wall, causing mechanical damage to the protoplasm. The dehydration of the cell can lead to intimate contact between protoplasm layers located opposite to one another. As water enters the cell during thawing, the joined walls separate. This is often accompanied by protoplasm separation from the walls, with damage happening to the protoplasm structure [2, 9, 10, 12]. The disruption of the structure of the object being frozen causes a dramatic decrease in its viscosity.

Long-term storage of the frozen starters at –10°C leads to the growth of larger crystals through a decrease in the size of smaller crystals (recrystallization). In turn, this causes a still severer structural disruption in the frozen object. The viscous starter cultures BBV displayed a larger decrease in relative viscosity than the nonviscous ones. Upon 6-month-long storage, the relative viscosity of BBV frozen in air decreased by a factor of 45, while that of the nonviscous cultures decreased by a factor of 25 and 16, respectively.

The smallest decrease in viscosity was observed for the starter cultures frozen at –45°C. Upon 6-month-long storage, the relative viscosity of the starter cultures frozen in air decreased by a factor 3.8 and that of the cultures frozen in the liquid coolant decreased by a factor of 2. This insignificant decrease in the liquid viscosity is due to the high freezing rate. Rapid cooling (to –25°C or –45°C) prevents considerable water and solute redistribution by diffusion and favors the formation of small, uniformly distributed ice crystals, thus causing the least possible damage to the structure of the object.

The genetic stability of the lactic acid microorganisms in the bacterial starter cultures frozen at different temperatures and under different conditions was evaluated in terms of preservation of morphological and biochemical properties and antagonistic activity, as well as in terms of the retention of the molecular weight of, and the number of fragments in, the DNA of the bacteria—information gained using genus-specific primers (16S for–16S rev) [7].

Cell morphology depends on many factors, including the culturing conditions, the age of the culture, and the composition of the medium. In the culturing of *L. bulgaricus* in the MRS Agar medium, the bacteria appear as bacilli, either separate or aggregated in chains. Figure 4 shows the micrographs of the lactic acid starter cultures recorded before their freezing and after their freezing and 6-month-long storage.

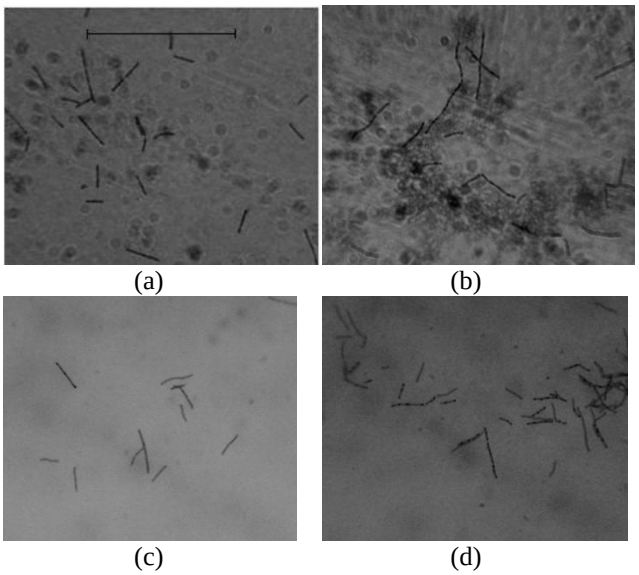


Fig. 4. Morphology of *L. bulgaricus* cells: (a) nonfrozen BBV, (b) nonfrozen BBNV, and (c, d) BBNV frozen and stored for 6 months at (c) –10 and (d) –45°C.

Considerable morphological changes were observed for all starter cultures frozen in air in all temperatures regimes and for the cultures frozen at –10°C in the liquid coolant. The microorganisms after freezing had curved cell walls, and most of then were separate. The BBNV starter cultures had lost their volutin granules, which were retained only in rare cases (Fig. 4c).

After freezing in the liquid coolant at –25 or –45°C, microorganisms with affected cell walls occurred sparsely, mostly in viscous starter cultures, and were separate or chained, and some of the bacilli had an elongated shape. Cells without volutin granules were observed in the BBNV cultures (Fig. 4f). Therefore, the freezing and low-temperature storage conditions were nondestructive.

We studied the antagonistic activity of *L. delbrueckii ssp. bulgaricus* strains contained in the starter cultures that had been frozen at –45°C in the liquid coolant and had been stored for 6 months. All of the strains retained a high antagonistic activity: the test bacteria growth suppression zone changed, on the average, by only 1–2mm (Table 4).

Table 4. Antagonistic activity of the *L. bulgaricus* cultures before and after freezing

RNCIM number	Growth suppression zone, mm (ε ± 1.0)				
	E. coli	*Sh. Flexneri*	*S. aureus*	*Proteus vulgaris*	*Proteus mirabilis*
B-3964	22/20	-/-	26/24	23/20	19/16
B-6516	13/11	12/10	9/7	19/18	17/15

In the study of the biochemical properties of the frozen bacterial starter cultures, it was observed that the saccharolytic activity of the lactic bacteria in the frozen

cultures was unchanged (Table 5).

Table 5. Biochemical properties of *L. delbrueckii ssp. bulgaricus* strains

Strain number	Cellobiose	Galactose	Lactose	Maltose	Mannitol	Mannose	Melibiose	Raffinose	Salicin	Sucrose	Trehalose	Arabinose	Sorbitol	Xylose	Aesculin	Melezitose
B-3964	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
B-6516	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–

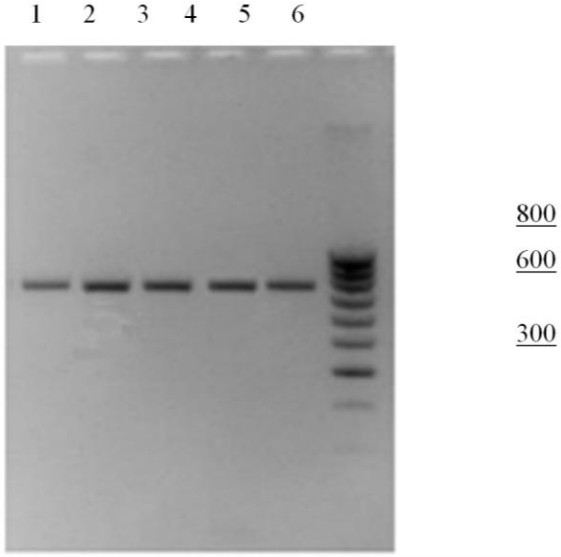


Fig. 5. Electrophoregram of the products of amplification of a DNA fragment of the 16S rRNA gene of *L. bulgaricus* bacteria: (1) B-6543, (2) B-6515, (3) B-6516, (4) B-3964, (5) B-3141, and (6) Medigene molecular weight marker.

We investigated the effect of freezing and lowtemperature storage on the stability of the DNA of five commercially valuable *L. delbruesckii ssp. bulgaricus* strains (B-3964, B-6516, B-3141, B-6543, and B-6515). Single-strain *L. delbruesckii ssp. bulgaricus* cultures were poured into 10-mL glass test-tubes under sterile conditions and were frozen at –45°C in the liquid coolant. The frozen starter cultures were stored in heat-insulated containers at –45°C for 6 months.

The DNA of the five *L. delbruesckii ssp.bulgaricus* strains was isolated via a procedure suggested by Bespomestnykh et al. [7]. Figure 5 shows the electrophoregram of the products of amplification of a DNA fragment of the 16S rRNA gene with the species-specific primers 16SbulF and 16SbulR for the five strains of the lactic acid bacteria frozen and stored for 6 months.

The electrophoresis data indicate that the molecular weight and the number of DNA fragments of the DNA of the bacteria are not affected by freezing and 6-month-long storage and, therefore, the lactic acid bacteria must have retained their morphological and biochemical properties.

These data suggest that temperatures of –25 to –45°C are optimal for freezing and storage of the starter cultures of the thermophilic lactic acid bacteria *L. bulgaricus*. After thawing, the starters that were stored under these conditions afford fermented milk products with a sufficiently dense clot, good organoleptic properties, and a high concentration of viable microorganisms.

REFERENCES

1. Rakhuba, D.V. and Novik, G.I., Kriokonservatsiya probioticheskikh mikroorgaizmov: nauchnye osnovy prakticheskogo ispol'zovaniya (Cryoconservation of probiotic microorganisms: scientific foundation of application), in *Mikrobnye biotekhnologii: Fundamental'nye i prikladnye aspekty* (Microbial Biotechnologies: Fundamental and Practical Aspects), Minsk: Loginov, 2007, issue 1, pp. 268–276.
2. *Kholodnyi stress i biologicheskie sistemy* (Cold Shock and Biological Systems), Tsutsaeva, A.A., Ed., Kiev: Naukova Dumka, 1991.
3. Sotchenko, O.G. and Safronenko, L.V., *Pishchevaya promyshlennost': nauka i tekhnologiya (Food industry: science and technology)*, 2009, no. 1 (3), pp. 14–18.
4. Sotchenko, O.G. and Danik, S.V., *Vestsi natsyyanal'nai akademii navuk Belarusi, seryya agrarnykh navuk (Bulletin of the national academy of science of Belarus, agricultural science series)*, 2005, no. 5, pp. 193–194.
5. Johnson, J.A.C. and Etzel, M.R., *Journal of Dairy Science*, 1995, vol. 78, no. 4, pp. 761–768.
6. Khamagaeva, I.S., Kachanina, L.M., and Tumurova, S.M., *Biotekhnologiya zakvasok propionovokislykh bakterii* (Propionic Acid Bacteria Starter Cultures: Production Biotechnology), Ulan-Ude: Vostochno-Sibirskii Gos. Tekhnol. Univ., 2006.
7. Bespomestnykh, K.V., Babich, O.O., Prosekov, A.Yu., and Korotkaya, E.V., *Tekhnika i teknologiya pishchevykh proizvodstv (Engineering and technology in the food industry)*, 2010, no 1, pp. 64–68.
8. Netrusov, A.I., Egorova, M.A., and Zakharchuk, L.M., *Praktikum po mikrobiologii* (Labs in Microbiology), Moscow: Akademiya, 2005.
9. Foschino, R., Fiori, E., and Galli, A.**,** *Journal of Dairy Research*, vol. 63, no. 2, pp. 295–303.

10. Fonseca, F., Marin, M., and Morris, G.J., *Applied and Environmental Microbiology*, 2006, vol. 72, no. 10, pp. 6474–6482.
11. Fonseca, F., Béal, C., and Corrieu, G., *Cryobiology*, 2001, vol. 43, no. 3, pp. 189–98.
12. *Kriobiologiya i biotekhnologiya* (Cryobiology and Biotechnology), Tsutsaeva, A.A., Ed., Kiev: Naukova Dumka, 1987

IDENTIFICATION AND PREVENTION OF THE FORMATION OF MEAT WITH PSE AND DFD PROPERTIES AND QUALITY ASSURANCE FOR MEAT PRODUCTS FROM FEEDSTOCKS EXHIBITING AN ANOMALOUS AUTOLYSIS BEHAVIOR

I. F. Gorlov*, E. I. Pershina, and S. L. Tikhonov*****

*Volga Research Institute of Production and Processing of Meat and Dairy Products,
Russian Academy of Agricultural Sciences,
ul. Rokossovskogo 6, Volgograd, 400131 Russia,
tel. (8442) 32-10-48; 32-11-01; e-mail: niimmp@mail.ru

**Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
e-mail: tovar_kemtipp@mail.ru

***Ural State Academy of Veterinary Medicine,
ul. Gagarina 13, Troitsk, Chelyabinsk oblast, 457100 Russia,
e-mail: tihonov75@bk.ru

Received February 14, 2013; accepted in revised form April 18, 2013

Abstract: A method for determining the stress resistance of slaughter animals by measuring the electric conductivity of the Tan-Fu biologically active point has been developed. The possibility of preventing the formation of signs of PSE and DFD properties in meat of stress-sensitive animals through the inclusion of succinic acid and a motherwort extract into their diet has been explored. A method for predicting the quality of meat of slaughter animals while they are alive has been developed. The possibility of using electrochemically activated water for proving consumer properties of meat products from feedstocks exhibiting an anomalous autolysis behavior has been explored.

Key words: meat with anomalies in autolysis, stress of slaughter animals

UDC 637.5
DOI 10.12737/2048

INTRODUCTION

At present, the problem of identification, prevention, and processing of meat feedstock exhibiting an anomalous autolysis behavior—the so-called meat with PSE (pale, soft, and exudative; pH < 5.2), DFD (dark, firm, and dry; pH > 6.4), and RSE properties (red, soft, and exudative; pH = 5.5)—is of high priority [1].

According to calculations of scientists, about 800 000 tons of DFD beef and 1 125 000 tons of PSE pork are used annually at domestic enterprises (the data are based on calculations of the NP Konsalting). It is obvious that the major portion of this meat makes the production of quality products problematic. For example, the use of PSE meat leads to an increase in the moisture loss during heat treatments and to the occurrence of a pale color, a sour taste, and an unusual texture of this type of product. Although DFD meat exhibits high moisture-retaining capacity (MRC) and water binding capacity (WBC), the use of this meat results in an increase in the duration of salting and the formation of a harsh texture of the product. Therefore, it is recommended that meat with signs of PSE properties in combination with DFD meat, plant protein preparations, and alkaline phosphates should be used in the uncooked smoked sausage technology. Meat with DFD properties is used in the production of cooked sausages, frozen ready-to-cook foods, and meat products with high yields.

The formation of meat with anomalies in autolysis is caused by a variety of factors and can occur during both production and processing [2, 3].

A significant effect on the probability of occurrence of defects is exerted by the fat content in the feedstock, which is true for both pork and beef. The probability of occurrence of DFD properties is high in beef containing less than 4% of muscle fat. Selective breeding of meat-type pigs aimed at changing the ratio between the muscle and fat tissues (from 1 : 1 to 1 : 0.5) has led to an increase in the amount of exudative and dense dry pork [4].

Another case of the formation of meat with a nonconventional quality is the violation of hormonal homeostasis of animals owing to hormonal stimulants (reproductive hormones and their counterparts in the form of synthetic hormone-like compounds) that are used to accelerate the growth and development processes and improve the digestibility of the feeding stuff.

One of the factors of the formation of meat with an abnormal quality is the lack of protein and minerals in the diet and physical inactivity in the case of an intensive technology of breeding and feeding of farm animals.

Meat with defects is largely determined by the genotype of the animal. The quality of meat significantly

varies depending on the breed and species of slaughter animals.

One of the main causes of the formation of meat exhibiting an anomalous autolysis behavior is the effect of stress factors on the body of slaughter animals. There are the following stress factors:

- Physical (temperature, humidity, solar and ionizing radiation, noise, air motion).
- Chemical (an increase in the concentration of ammonia, hydrogen sulfide, carbon dioxide, and other gases in the air; chemicals used in livestock breeding and crop production).
- Psychic (ranking stresses in the struggle for dominance in a group of animals kept in loose housing systems to establish a hierarchical order and determine the leader of the group).
- Transport (loading and unloading, transportation of animals by various means of transport).
- Technological (weighing, weaning young stock from their mothers, crowded housing, small fronts of feeding and watering, mistreatment of animals, increased noise from the operation of machinery, long-term drives, sharp changes in housing conditions, etc.).

The above stress factors, or stressors, can be described as unfavorable housing conditions that can cause a stress under which the hormonal homeostasis of animals changes [5]. Farm animals of the same breed and species respond differently to the effects of stress factors. Animals are divided into sensitive and resistant to stress factors [5, 6]. Stress-sensitive animals are characterized by meat with PSE and DFD properties.

In view of the above, the current focus of research in the field of quality of meat products is the development of methods for determining the stress resistance of animals, the prevention and reduction of the negative effects of stresses on stress-sensitive animals, the development of reliable methods for identifying meat into groups with PSE and DFD properties, and, accordingly, quality assurance for meat products from feedstocks exhibiting an anomalous autolysis behavior.

The aim of this study is to prevent the formation of meat feedstock with defects in stress-sensitive animals, to identify meat with an anomalous autolysis behavior, and to improve the processing characteristics of meat systems.

To implement this objective, it is necessary to solve the following problems:

- To develop a method for determining the stress resistance of slaughter animals.
- To prevent the formation of meat with signs of PSE and DFD properties under the action of stress factors on stress-sensitive animals.
- To develop a method for identifying meat with an anomalous autolysis behavior.
- To explore the possibility of using electrochemically activated (ECA) water to provide a high quality of products from feedstocks exhibiting an anomalous autolysis behavior.

MATERIALS AND METHODS

The following objects were studied:

- slaughter animals (bull-calves of a black-and-white breed);
- beef (the shoulder portion of the carcass and longissimus dorsi muscle after aging for 24 h);
- ECA water (anolyte);
- "Russkaya" cooked sausage samples.

Organoleptic, physicochemical, biochemical, microbiological, and instrumental methods of research were used.

The blood glucose level was determined by an orthotoluidine method based on the interaction between the aldehyde group of glucose and the amino group of orthotoluidine in an acid environment with the appearance of a blue-green color.

The sampling was conducted according to GOST (State Standard) R 51447-99 (ISO 3100-1-91) "Meat and meat products: Sampling methods."

The organoleptic analysis was conducted in accordance with GOST (State Standard) 7269-79 "Meat: Sampling methods and organoleptic methods for determining the freshness."

The pH value was determined by the potentiometric method according to GOST (State Standard) R 51478-99 (ISO 2917-74) "Meat and meat products: A test method for determining the hydrogen ion concentration (pH)."

The glycogen content was determined from the color reaction with anthrone, which is based on the hydrolysis of proteins with an alkali, the isolation of glycogen from the solution with ethyl alcohol, its washing and dissolving, reaction with anthrone, and the development of color with the subsequent measurement of its intensity.

The content of lactic acid was determined through the sedimentation of proteins and carbohydrates, heating with sulfuric acid, the development of a color reaction with veratrole, and measurement of the color intensity.

The WBC of meat was studied from the amount of bound water by the Grau–Hamm press-method in the modification of the All-Russia Research Institute of Meat Industry (VNIIMP).

The safety of the product was estimated in accordance with SanPin 2.3.2.1078-01 "Hygienic requirements for the safety and nutritional value of food products."

RESULTS AND DISCUSSION

A few methods for determining the stress resistance of farm animals are currently used. Thus, the stress resistance of cows is estimated from the pattern of milk ejection during machine milking; this parameter for pigs is determined according to the response of the animals after subcutaneous injection of turpentine. In addition, the stress resistance of cattle is determined under loading with adrenocorticotropic hormone (Thorn test). Preliminarily, the amount of eosinophils in the blood is counted; after that, adrenocorticotropic hormone is intramuscularly injected in a dose of 25 units per 100 kg of live weight, and the amount of eosinophils in the blood is counted again after 2 and 4 h. A decrease in the number of cells by more than 50% and the failure to return to the initial state after 4 h suggest that the animal is highly sensitive to stresses.

A generally accepted method for determining the stress resistance of bull-calves is based on the level of cortisol in the blood. Animals with high stress resistance exhibit a rapid normalization of homeostasis: even in 30 min after the occurrence of stress reaction, the

amount of cortisol returns to the initial level; for animals with low stress resistance, a high content of plasma cortisol is observed for a long time.

We have developed a method for determining the stress resistance according to the electric conductivity of the Tan-Fu biologically active point (BAT) responsible for the activities of the cardiovascular system, because it is the most sensitive to the impact of stress factors.

Animals with a difference of potentials in the BAT of less than 5 μA are regarded as stress-resistant. Animals exhibiting a difference of potentials in the Tan-Fu BAT of 2–4 μA are stress-adaptable. Animals having this parameter at a level of more than 4 μA are stress-sensitive.

Tests of 95 bull-calves of a black-and-white breed at the age of 18 months have revealed that 46 animals (48%) are stress-sensitive, 28 animals (30%) are stress-adaptable, and 21 animals (22%) are stress-resistant. The novelty of the engineering solution is protected by RF patent no. 2 292 197 "A method for determining the stress resistance of bull-calves" [6].

The next stage of our studies was the prevention of the formation of meat with an anomalous autolysis behavior caused by a transport stress of stress-sensitive animals.

For the experiment, two groups of ten stress-sensitive slaughter animals in each were formed (bull-calves of a black-and-white breed at the age of 16 months).

The animals were transported to a distance of 110 km. Before the transportation, the bull-calves of the first group received their basic diet; the basic diet of the animals of the second group was enriched in succinic acid a dose of 100 mg/kg, and a motherwort herb extract in an amount of 50 mg/kg in the form of a 10% aqueous solution was injected into these animals during 10 days prior to transportation. The motherwort extract was manufactured at the OOO NPP Erakond-Ural according to the technology for producing extracts from plant raw materials (RF patent no. 2 456 825).

During transportation, the animals were in a state of stress: the body temperature, respiratory rate, and cardiac rate were increased. Table 1 shows the clinical data for the animals subjected to tests.

The results of examination of the clinical data under stress are consistent with the results of foreign authors [7–9].

It is evident from Table 2 that the introduction of a motherwort extract and succinic acid into the diet of slaughter animals reduces the live weight losses under transport stresses (Table 2).

The results of studying the quality of meat of the stress-sensitive bull-calves of the control group and the group treated with succinic acid and a motherwort extract are shown in Table 3.

The deep brown color of the meat of the stress-sensitive animals of the first group is caused by the mechanism of stress development. Thus, at the stage of anxiety, or the stage of mobilization (this is the first short-term phase of the stress), the secretion of adrenaline increases, which enhances the heart activity. Red blood cells are released into the bloodstream to help the respiratory system to absorb oxygen. Therefore, if animals are in a state of stress during slaughter, then a higher amount of hemoglobin remains in their meat, which subsequently leads to the formation of a dark color.

Table 1. Clinical and physiological data of stress-sensitive bull-calves of the control group and the group treated with succinic acid and a motherwort extract (X ± mx; *n* = 10)

Group	Temperature, °C	Pulse, beats per minute	Respiratory rate per minute
	Before transportation		
1	37.70 ± 0.02	68.5 ± 2.2	29.4 ± 1.5
2	37.70 ± 0.05	67.3 ± 2.5	28.7±1.3
	In 1 h after transportation		
1	38.30 ± 0.04	76.3 ± 2.1*	35.3 ± 1.0**
2	37.90 ± 0.03	75.0 ± 2.4*	32.2 ± 1.8
	In 24 h after transportation		
1	38.2 ± 0.02	75.1 ± 1.9**	30.5 ±1.6
2	37.70 ± 0.04	68.4 ± 2.5	28.0 ± 1.5

Hereinafter, the value is significant: * at $P < 0.05$; ** at $P < 0.01$; and *** at $P < 0.001$.

Table 2. Losses of live weight of bull-calves of the control group and the group treated with succinic acid and a motherwort extract (X ± Sx; *n* = 10)

Parameter	Group of animals	
	1	2
Body weight of animals before transportation, kg	341.0 ± 2.5	340.3 ± 2.1
Body weight of animals after transportation, kg	318.2 ± 3.1***	329.4 ± 3.2**
Live weight losses during transportation, kg	22.8	10.9
Live weight losses, %	6.7	3.2

A motherwort extract regulates the functional state of the central nervous system, decreases the secretion of adrenaline and thereby has a calming effect and reduces the nervous irritability during stress. Therefore, the animals of the second group were less susceptible to the effects of stress factors; their hormonal status was normal; this subsequently had a positive impact on the organoleptic parameters.

During stress, the functional activity of the anterior pituitary and adrenal cortex increases and the secretion of adrenocorticotropic hormone (ACTH) increases manifold; under the action of thereof, the incretory activity of the adrenal cortex enhances, which contributes to a rapid arrival of corticosteroid hormones in the bloodstream. Glucocorticoids and mineralocorticoids (they are also referred to as adaptive hormones) complement each other and are involved in the organization of a protective response. During stress, glucocorticoids increase the blood glucose level. However, the stores of carbohydrates in the body are limited and rapidly consumed. Therefore, before transportation, the animals received succinic acid as a source of energy.

Table 3. Quality of meat of the stress-sensitive bull-calves of the control group and the group treated with succinic acid and a motherwort extract (X ± Sx; *n* = 10)

Parameter	Group of animals	
	1	2
Color	Deep brown	Red-pink
Texture	Elastic, dense. A pit formed under pressing with a finger levels out slowly.	Elastic, dense. A pit formed under pressing with a finger levels out rapidly.
Flavor of meat	Specific, characteristic of fresh meat	Specific, characteristic of fresh meat
Cooking test - flavor of broth - transparency - flocculation	 Aromatic Turbid Significant	 Aromatic Transparent Insignificant
Degree of bleeding	Satisfactory	Good
pH	6.5 ± 0.1	5.8 ± 0.1*
Glycogen, mg%	12.1 ± 2.7	860.1 ± 15.7***
Lactic acid, mg%	118.4 ± 12.0	948.1 ± 10.1*
WBC, %	67.9 ± 3.5	61.1 ± 2.0

It has been found that the amount of the energetic material—glycogen—in the meat of the bull-calves of the second group was significantly higher. The decomposition of glycogen during anaerobic glycolysis yields lactic acid, which leads to a shift of pH to the acidic side. The content of lactic acid in the meat samples of the first and second groups was at a level of 118.4 and 948.1 mg%, respectively.

The pH value characterizes the level of ionization of the amino groups of the protein molecule and predetermines the ratio between charged and uncharged groups, which affects the amount of hydrophilic centers and, accordingly, the WBC value. The last-mentioned quantity changes the rheological properties, organoleptic parameters, structure, and degree of stability of meat products during storage.

The results of the studies have shown that, in 24 h after slaughter, the pH value of the meat was 6.5 for the bull-calves of the first group and 5.8 for the animals of the second group against the background of the use of succinic acid and a motherwort extract. The beef samples obtained from the first group of bull-calves had a high WBC (67.9%), which is characteristic of meat feedstocks with DFD properties.

Thus, the treatment of stress-sensitive slaughter animals with succinic acid and a motherwort extract hinders the formation of meat with anomalies in autolysis.

The most important criteria for estimating the meat exhibiting an anomalous autolysis behavior are organoleptic biochemical parameters and processing properties.

A significant contribution to the identification of meat feedstocks into quality groups was made by foreign scientists Eikelenboom, Smuldes, Ruderus, Hawrysh, Shand, Wolfe [10, 11], etc.

One of the methods for controlling the nutritional value of meat involves the butchering of animals, the sampling of the test specimen, and the subsequent control of the nutritional value of meat by sorting carcasses into groups of meat with a normal nutritional value (NOR) and PSE and DFD properties. The gist of this method is that, after the bleeding of carcasses (during butchering), the free activity of tissue proteinase—cathepsin D—is determined in the muscle tissue; meat with the cathepsin D activity of less than 0.05 μM/h • g of protein is regarded as meat with a normal nutritional value; the cathepsin D activity of 0.05–0.075 μM/h • g of protein is characteristic of meat with DFD properties; and the cathepsin D activity of more than 0.075 μM/h • g of protein is typical for meat with PSE properties [12].

Another method consists in the exposure to an electric current and the measurement of the pH value. Yet another method is based on the sampling of the test specimen, the exposure to electromagnetic radiation of a given wavelength range, and the measurement of the parameter that correlates with the quality of meat, i.e., the ratio between reflection intensities of the test specimen and the reference, which are measured using a color comparator (KTsSh). In this case, the quality of meat is controlled with allowance for the derived values of this ratio. The ratio of 1.05–1.0 characterizes NOR meat; the ratio of 1.2–1.25 is typical for DFD meat; and the ratio of 0.9–0.95 is characteristic of PSE meat [12].

The generally accepted method for classifying meat feedstocks into groups of PSE, DFD, and NOR quality is based on the measurement of pH values in 1 and 24 h after the slaughter of the animal [13].

The method developed in the United Kingdom makes it possible to classify meat into quality groups according to the scattering of light passing through the probe placed in the meat.

Canadian researchers use a portable spectrophotometer to derive reflection spectra in a wavelength range of 400–700 nm. The measurement data correlate with the quality factors for meat with DFD and PSE properties.

Polish specialists have developed a method for determining the quality of beef in slaughter lines, which is based on the rapid glycolysis of meat. In this case, 3 g of the muscle tissue is mixed with 20 mL of calcium chloride and 20 mL of magnesium chloride; after that, the pH value is measured to classify meat into quality groups [3].

Another method for identifying meat with a nonconventional quality is based on the measurement of the electric conductivity of biologically active points of animals while they are alive [14].

Each of the above methods has certain disadvantages, and their practical use is fairly limited. Therefore, the development of reliable and affordable methods is an urgent problem.

We have developed a method for predicting the quality of meat of slaughter animals while they are alive. The method consists in the following. The blood glucose content of animals is determined at the beginning of the preslaughter holding; after that, they are given to drink a 10% glucose solution in a dose of 2 L per 100 kg of live weight, and the analysis is repeated after 24 h. If the measured value is higher than the initial one, then it is concluded that the animal is prone to

the formation of meat with anomalies in autolysis.

To verify the method, an experiment was conducted. Two groups of 20 slaughter animals in each were formed (bull-calves of a black-and-white breed at the age of 16 months). The first group was a control group. At the beginning of the preslaughter holding, the animals of the second group were given to drink a 10% glucose solution in the above dose. Within 24 h of the preslaughter holding, the glucose content in the control group significantly decreased to 2.7 mmol/L compared to a value of 3.1 mmol/L at the beginning of the preslaughter holding; this is attributed to the fact that the animals were not fed. In the test group, the glucose content returned to the initial level of 2.3–3.9 mmol/L for ten animals, became higher—3.9–4.13 mmol/L—for six animals, and became lower than the initial level for four animals.

The results of the post-slaughter measurement of pH in the longissimus dorsi muscle are shown in Table 4.

Table 4. Classification of meat into quality groups

Number of animals	Blood glucose content, mmol/L	pH of meat in 45 min after slaughter	Quality group of meat
4	2.14–2.2	5.3–5.7	PSE
10	2.3–3.9	5.3–6.2	NOR
6	3.9–4.13	6.4–6.6	DFD

After 45 min, the pH of the meat of the slaughter animals with a low glucose content (2.14–2.2 mmol/L) was 5.3–5.7, which is indicative of meat with PSE properties; six animals had a pH value of more than 6.4 (meat with DFD properties), and only ten animals had a normal pH value.

Thus, the use of the proposed engineering solution makes it possible to classify the quality of meat of slaughter animals while they are alive into three groups—PSE, DFD, and NOR—by treating the slaughter animals with a 10% solution of glucose and measuring its content in their blood.

It is recommended that meat with DFD properties should be used in the production of cooked sausages. It should be noted that meat products from this meat feedstock are unstable to microbiological spoilage because of high WBC and pH values.

To provide a high quality of meat products, phosphates of different brands are used, in particular, "Albright," "Puromix," and "Puron," which give the possibility to compensate for the defects of meat feedstock (PSE and DFD).

Therefore, the providing of a high quality of meat products during storage is of current concern.

The shelf life of cooked sausages in natural casings is up to 5 days at a storage temperature of 0–6°C and a relative air humidity of no high than 75%.

Studies on the use of ECA water for the production of cooked sausages from meat feedstock with signs of DFD properties were conducted.

For the experiment, an experimental batch of the "Russkaya" cooked sausage was produced according to GOST (State Standard) R 52196-03. The shelf life of the sausage is 5 days. The first group (control) was produced according to the conventional technology and formulation, while the second group of the sausage samples was prepared using ECA water.

The sausage samples were placed in a storage room (t° of 0–4°C; relative air humidity of 75%).

The storage ability of the sausage was determined from organoleptic and microbiological parameters, peroxide value, pH value, and the results of microscopic studies.

ECA water (anolyte with a pH value of 2.5 from drinking water) was used for the preparation of the cooked sausages. The use of the anolyte in the production of cooked sausages is attributed to a high pH value of the meat feedstock.

Figure 1 shows the results of the organoleptic estimate of the control and test samples of the cooked sausages after storage for 5 days (in points).

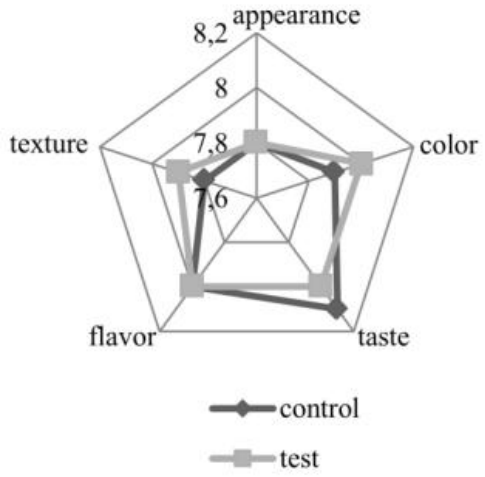


Fig. 1. Organoleptic estimate of the cooked sausages after storage for 5 days (in points).

It is evident from Fig. 1 that the test samples of the cooked sausages were superior to the control samples in color and texture.

After storage for 5 days, the sausage samples of the control and test groups met the requirements of SanPiN 2.3.2 1078-01.

Table 5 shows the organoleptic parameters of the cooked sausages after storage for 10 days.

The organoleptic studies have revealed that, after 10 days, the sausage samples of the control group had an unpleasant taste and odor and a gray-green color. The sausage filling of the product was softened and loose. All the examined organoleptic parameters of the sausage of the test group were within normal limits.

One of the important criteria of the freshness of meat products is the pH value.

It has been found that the sausages of the first group, after storage for 3 and 5 days, have a pH value of 5.2 and 5.8, while the normal value for a fresh product is 5.0–6.8; after 10 days, their pH is 7.0, which corresponds to sausages of dubious freshness. This is probably attributed to the accumulation of metabolic products of microorganisms that cause the breakdown of proteins (amines, nitrogen bases, ammonia). After storage for 10 days, the medium's reaction of the test cooked sausage samples is at a level of 6.4.

Table 5. Organoleptic parameters of the cooked sausages after storage for 10 days

Parameter	Requirements of GOST R 52196-2003	Group	
		1 (control)	2 (test)
		Actual	
Outward appearance	Loaves with a clean dry surface free from stains, slips, casing damage, sausage filling overflows, broth and fat pockets	Loaves with a moist surface and sticky casing	Loaves with a clean dry surface free from stains, slips, casing damage, sausage filling overflows, broth and fat pockets
Texture	elastic	The sausage filling of the product is softened and loose.	elastic
Cross sectional view of the sausage filling	The sausage filling is light pink to dark pink in color with no gray stains; it is homogeneous, uniformly mixed and has inclusions of lard no larger than 6 mm.	The sausage filling taken from the surface of the loaf is light pink to greenish in color with gray stains; it is homogeneous, uniformly mixed and has inclusions of lard no larger than 6 mm.	The sausage filling is light pink to dark pink in color with no gray stains; it is homogeneous, uniformly mixed and has inclusions of lard no larger than 6 mm.
Taste and flavor	Pleasant, characteristic of this type of product, free from foreign flavor and odor, with a pronounced flavor of spices.	Unpleasant odor, slightly sour taste	Pleasant, characteristic of this type of product, free from foreign flavor and odor, with a pronounced flavor of spices.

An increase in the peroxide and acid values of the sausages during storage was not observed.

One of the main causes of spoilage of meat products is the development of residual microflora in sausage products during storage.

Table 6 shows the microbiological parameters of the cooked sausages after storage for 10 days.

Table 6. Microbiological parameters of the cooked sausages after storage for 10 days

Parameter	Norm according to SanPuN 2.3.2 1078-01	Group	
		1 (control)	2 (test)
QMAFAnM, CFU/g, no more	1 x 10^3	15 x 10^6	15 x 10^1
CGB (coliforms) in 1.0 g	not allowed	not detected	not detected
sulfite-reducing clostridia in 0.01 g	not allowed	not detected	not detected
S.aureus in 1.0 g	not allowed	not detected	not detected
Pathogenic, particularly salmonellas, in 25 g	not allowed	not detected	not detected

After storage for 10 days, the control cooked sausage samples do not meet the regulatory requirements. Thus, the amount of QMAFAnM is 15 x 10^6 CFU/g, while the norm is no more than 1 x 10^3 CFU/g; hence, the studied product is unfit for sale and consumption. At the same time, the test cooked sausage samples do not exhibit deviations in the microbiological parameters.

Analysis of the microbiological studies suggests that the use of ECA water in the production of cooked sausages constrains the development of pathogenic microflora in the product.

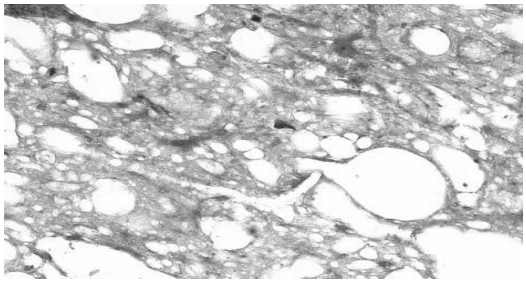


Fig. 2. Microstructure of the cooked sausage samples after storage for 5 days (control).

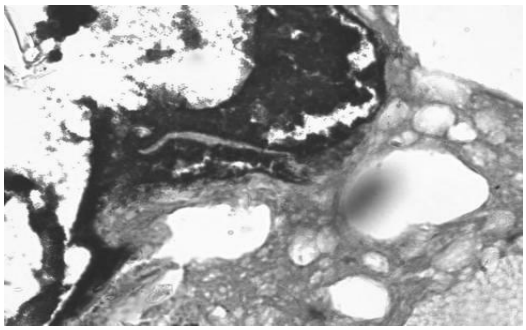


Fig. 3. Microstructure of the cooked sausage samples after storage for 10 days (control).

The microstructure of the cooked sausage during storage was studied. Microscopically, the sausage is a homogeneous amorphous finely grained mass with large particles of fat, dispersed inclusions of fibers of the transversely striated muscle tissue, and fragments of loose and dense connective tissue.

Figure 2 shows the cooked sausage of the control group after storage for 5 days.

A cluster of cocci and colonies of yeast fungi are evident in Fig. 3.

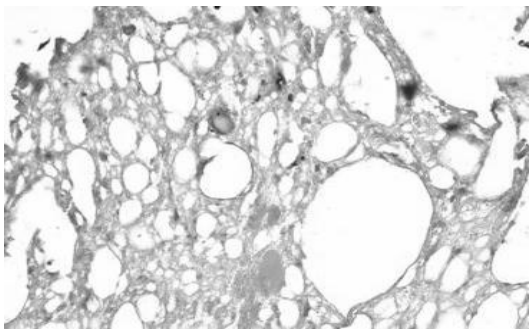


Fig. 4. Microstructure of the cooked sausage samples after storage for 5 days (test).

Thus, on the basis of the studies, a method for determining the stress resistance of slaughter animals has been developed. It has been shown that the meat of stress-sensitive bull-calves under transport stress is characterized by DFD defects. The use of succinic acid and a motherwort extract in the diet of slaughter animals before transportation makes it possible to hinder the formation of meat with a nonconventional quality. A method for predicting meat with PSE, DFD, and NOR properties has been described. It has been shown that ECA water can be used in the production of meat products from feedstocks exhibiting an anomalous autolysis behavior.

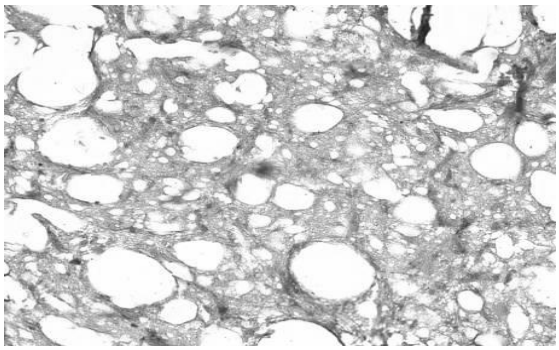


Fig. 5. Microstructure of the cooked sausage samples after storage for 10 days (test).

All the studied pieces (Figs. 4, 5) contain individual colonies of cocci. The fragments of the connective tissue exhibit the monotony of perception of histological stains; however, the nuclei are clearly visible.

Thus, the use of ECA water in the production of cooked sausages has a positive effect on the shelf life of products from feedstocks with DFD properties.

REFERENCES

1. Rogov, I.A., Zharinov, A.I., Tekut'eva, L.A., and Shepel', T.A., *Biotekhnologiya myasa i myasoproduktov* (Biotechnology of Meat and Meat Products), Moscow: DeLi print, 2009.
2. Krishtafovich, V.I., Kolobov, S.V., and Yablokob, D.I., Potrebitel'skie svoistva myasa s otkloneniyami v protsesse avtoliza (Consumer properties of meat with anomalies in autolysis), *Myasnaya Industriya* (*Meat Industry*), 2005, no. 1, pp. 30–33.
3. Zharinov, A.I. and Kudryashov, L.S., Chto nuzhno znat' o parnom myase (What we should know about slaughter-warm meat), *Myasnaya Industriya* (*Meat Industry*), 2005, no. 7, pp. 16–19.
4. Tatulov, Yu.V., Kosacheva, T.V., and Kuznetsova, S.A., Vliyanie stressa na kachestvo myasnogo syr'ya (Effect of stress on the quality of meat feedstock), *Myasnaya Industriya* (*Meat Industry*), 2009, no. 7, p. 55.
5. Tikhonov, S.L., *Stress i kachestvo myasa* (Stress and Quality of Meat), Troitsk: UGAVM, 2007.
6. Tikhonov, S.L., Tikhonova, N.V., Sunagatullin, F.A., and Stepanov, A.V., RF Patent 2 292 197, 2005.
7. Everly, G.S., The organ specificity score as a measure of psycho-physiological stress reactivity, *Doctoral Dissertation*, University of Maryland, 1978 (unpublished).
8. Levi, L., Stress, distress, and psychosocial stimuli, in *Occupational Stress*, McLean, A., Ed., Springfield, Ill.: Thomas, 1974.
9. Vigas, M., in *Catecholamines and Stress: Recent Advances, Ser. Developments in Neuroscience*, Usdin, E., Kvetnansky, R., and Kopin, I.J., Eds., New York, Amsterdam, Oxford: Elsevier, 1980, vol. 8, pp. 573–578.
10. Eikelenboom, G., Smuldes, F.J.M., and Ruderus, H., The effect of high and low voltage electrical stimulation on beef quality, *Meat Science*, 1985, vol. 15, no. 4, pp. 247–254.
11. Hawrysh, Z.J., Shand, P.J., and Wolfe, F.N., Effects of low voltage electrical stimulation on the quality of the round from young and mature cows, *Canadian Institute of Food Science and Technology Journal*, 1986, vol. 19, no. 3, pp. 121–129.
12. Kudryashov, L.S., Gurinovich, G.V., and Potipaeva, N.N., RF Patent 2 092 836, 1997.
13. Tatulov, Yu.V., Nemchinova, I.P., and Loseva, N.A., Kachestvennye kharakteristiki syr'ya myasnoi promyshlennosti v SSSR i za rubezhom (Quality characteristics of feedstock for meat industry in the USSR and abroad), in *Myasnaya i kholodil'naya promyshlennost': Peredovoi nauchno-proizvodstvennyi opyt, rekomenduemyi dlya vnedreniya* (Meat and Refrigeration Industry: Advanced Research and Production Experience Recommended for Implementation), Moscow: AgroNIITEImyasomolprom, 1990, issue 2, pp. 22–26.
14. Lapshina, A.A., Tikhonov, S.L., Pershina, E.I., and Kudryashov, S.L., Sposob sortirovki myasa na gruppy kachestva PSE, DFD i NORM (A method for sorting meat into PSE, DFD, and NORM quality groups), *Myasnaya Industriya* (*Meat Industry*), 2012, no. 9, pp. 24–27.

REGULARITIES OF THE DRYING OF LACTULOSE SOLUTIONS

O. V. Kozlova*, A. I. Piskaeva*, V. F. Dolganyuk*, and B. G. Gavrilov**

*Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone/fax: +7 (3842) 39-68-88, e-mail: kozlovaov79@rambler.ru

**The Yaroslavl State Institute of Quality of Raw Materials and Foodstuff,
Moskovskii pr. 76 a, Yaroslavl, 150030 Russia,
phone/fax: +7 (4852) 44-74-84, e-mail: Milkyar@mail.ru

Received April 3, 2013; accepted in revised form October 15, 2013

Abstract: The existing patented technologies of the production of lactulose were analyzed, and leading producers were defined. Crystalline lactulose was produced via the spray drying of lactulose solutions with various mass fractions of solids. The principal dependences of the finished product output on the drying temperature, the solution flow rate, the air flow rate generated by an aspirator, and the gas spray rate were studied. The results of analyzing the presented dependences allowed us to determine the optimal mass fraction of solids in a solution for spray drying. The results of studying the quantitative parameters of dry lactulose, including hygroscopicity, particle size, moisture content, and finished product solubility index, were presented.

Key words: lactulose, lactose, drying, solution, temperature, solution flow rate, air flow rate, mass fraction, solubility index

UDC 637.345:664.9.047
DOI 10.12737/2049

INTRODUCTION

Lactulose is a carbohydrate that belongs to the class of oligosaccharides and the subclass of disaccharides. Its molecule consists of the galactose and fructose moieties. Lactulose is obtained from milk sugar (lactose) via the deep processing of milk. Lactulose is a white odorless sweet crystalline substance, which is well soluble in water [1].

Lactulose is a carbohydrate, which is indigestible in the gastrointestinal tract, but usable by beneficial microflora as a nutritious substrate [2].

The bifidogenic properties of lactulose were first revealed and studied by F. Petuely, a British pediatrician, who has succeeded in separating lactulose from human breast milk and also in establishing its beneficial effect on the destroyed microbiocenosis of the gastrointestinal tract of artificially fed babies and their general health [3].

Lactulose is currently classified as a classic prebiotic. The general principle of its effect consists in reducing the amount of pathogenic bacteria, such as *Escherichia coli* and *Staphylococcus*. Moreover, lactulose stimulates the growth of probiotic bacteria, such as *Bifidobacterium*, which are known to produce a positive effect [4].

According to research data, the world production of lactulose preparations is currently 20 000 t/year, and over 5% of produced lactulose is used for this purpose. The assortment of lactulose preparations includes over ten items. Japan alone produces three types of syrups and two types of crystalline lactulose that satisfy the international quality standard [5].

The leading company in the world market of lactulose is Morinaga Milk Industry (Japan) dealing with the problems of studying the properties of lactulose and the development of methods for its production since 1953, i.e., for more than 60 years.

The market of lactulose in the Russian Federation has generally been represented by the pharmaceutical preparations of foreign production until now. OOO Shekhon-Laktuloze that produces lactulose syrup with a mass fraction of solids of no less than 66% is recognized to be the only large-capacity producer of lactulose in the Russian Federation.

It is considered that dry lactulose powders are more technological in use [5]. However, the problem of the production of crystalline lactulose has not completely been solved in Russia until now. However, a number of technologies have already been patented.

According to patent data, the key problem in the existing technologies of the spray drying of lactulose solutions is the presence of binders, and the amorphous powder obtained at the outlet has high hygroscopicity. The analysis of the existing patented technologies and parameters of the drying of lactulose solutions is given in Table 1.

The analysis of the performed patent studies shows that among the disadvantages of the production of dry lactulose preparations by spray drying is a lowered content of lactulose in a product due to different catalysts and binders that are added to reduce hygroscopicity and accelerate the process. The growth of price due to the incorporation of fillers is also noteworthy.

Table 1. Technologies and parameters of the drying of lactulose solutions with various mass fractions of solids

Patent name	Drying parameters			
	technology	temperature, °C	time, h	auxiliary components
A process for the production of lactulose powder (United States) [6]	Spray drying	80–150	–	0.3-% cOPPUAkY solution
A method for the production of lactulose-containing powder and its application in fodders. A method for the production of lactulose-containing powder (Japan, Great Britain) [7, 8]	Spray drying	95–140	2–3	Potassium hydroxide
A method for the purification of lactulose syrup (Japan) [9]	Lyophilic drying	90–120	4–5	–
A method for the separation of lactulose. A method for the production of non-hygroscopic lactulose-containing powder (Japan, United States) [10, 11]	Vacuum drying	below 80	1	Ethanol
A composition on the basis of plant fibers and lactulose (United States) [12, 13]	Spray drying	60–90	6	Alginates
A method for the production of solid lactulose (United States) [14]	Solution inspissation, milling, crushing	80–100	5–7	–
A method for the production of crystalline lactulose (Unites States) [15]	Vaporization, cooling, crystallization	50–60	100–120	Lactulose trihydrate

The methods of the production of crystalline lactulose forms are very laborious, so the prices of preparations grow by 1–2 orders of magnitude in comparison with syrups. For the reason of the abovesaid, it is topical to study the spray drying of lactulose solutions with the purpose of establishing the optimal parameters, which will help us to obtain a product with high qualitative characteristics without fillers. The fact that import preparations have a rather high price in addition to "nature-identical" ingredients will provide the competitiveness of a product.

In this connection, we have studied the spray drying of lactulose solutions.

OBJECTS AND METHODS OF STUDY

At different stages of our work, the objects of study were (1) solutions with a mass fraction of lactulose of 20–60%; and (2) dry lactulose with a mass fraction of lactulose of 40%.

They were prepared from a solution with a mass fraction of lactulose of 50% via its dilution to 20–40% and additional inspissation to obtain a solution with a mass fraction of lactulose of 60%.

The solutions were dried on a Mini Spray Dryer B-290 setup (BUCHI Labortechnick AG) with the possibility of adjusting the flow rate of a working solution and the velocity of a spraying flow. The setup allows the production of a finished product with a particle size of 1–25 μm. The materials that come into contact with a product are acid-proof stainless steel, borosilicate glass, and silicone. The technical characteristics of the drier are given in Table 2.

The directions of the sprayed sample and the drying air coincide in the standard operational mode of the dryer. The pressure in the system is subatmospheric to prevent the contamination of a product in the case of leaks.

The spray drying setup is schematized in Fig. 1. The heating of the supplied gas and two-flow nozzle *1* are controlled by Fuzzy-Logic microprocessor automatic device *2* with a digital display and a PT-100 temperature detector, which provides a high accuracy of temperature adjustment. The solution is passed through the nozzle spraying it into smallest drops and enters chamber *3*, in which the process of drying occurs. The particles entrained by the gas flow move into cyclone *4*, where they are separated under gravity. The setup is equipped by filter *5* that rejects small particles and aspirator *6* that creates the air flow. The view of the setup is shown in Fig. 2.

Table 2. Technical characteristics of the experimental spray drying setup

Characteristic	Value
Voltage	200/230 V; 50–60 Hz
Maximum air flow rate	35 m^3/h
Motor control	Frequency transducer
Maximum inlet temperature	220°C
Heating power	2300 W
Heating control	PT-100; Fuzzy Logic; temperature control accuracy, ±3°C
Serial interface	RS-232 for the transmission of all parameters
Sprayed gas	Compressed air, 200–1000 dm^3/h, 5–8 bar
Nozzle hole diameter	0.7 mm
Nozzle cap diameter	1.4 and 1.5 mm
Average residence time	1.0–1.5 s
Dimensions, width × length × height	60 × 50 × 110 cm
Weight	48 kg

The mass fraction of solids in lactulose solutions was determined refractometrically according to GOST (State Standard) 24908-84.

The mass fraction of lactulose and other carbohydrates in solutions and dry lactulose was determined by gas-liquid chromatography (GLC), as it is an available and well-proven technique of the analysis for carbohydrates. It is based on the conversion of saccharides into volatile trimethylsilyl derivatives and their subsequent

separation on a GLC column and analysis with a flame ionization detector.

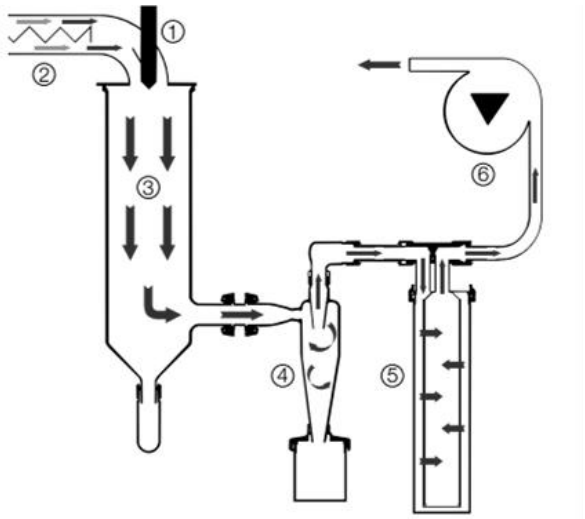


Fig. 1. Scheme of the spray drying setup: (*1*) nozzle, (*2*) heater, (*3*) spray chamber (cylinder), (*4*) cyclone, (*5*) filter, (*6*) aspirator.

Fig. 2. Spray drying setup.

The essence of the methods consists in the following. An analyzed sample after preliminary drying and skimming is treated with *N*-trimethylsilylimidazole at a temperature of 60–70°C for 1–2 h. A precisely dosed volume of hexane, the excess of which is hydrolyzed with water, is then added to the mixture, thereupon a hexane phase aliquot is injected into a chromatograph. Carbohydrates are separated on a packed column with a polar phase in the isothermal regime. Monosaccharides go out with solvent background, lactose goes out in the form of two peaks corresponding to α- and β-anomers, and lactulose goes out in the form of a single peak.

The content of lactulose and lactose was calculated by the inner standard method with precalibration.

The quality of the finished product was estimated by such characteristics as particle size, moisture content, solubility index, and hygroscopicity.

The solubility index was determined in compliance with GOST (State Standard) 30305.4.95.

The particle size was determined via the microscopy of a lactulose sample on an AxioVert.A1 inverted microscope (Carl Zeiss AG) with a magnification of X40.

The moisture content was determined using a Chizhova instrument.

The operating principle of the instrument consists in exsiccation via the vaporization of a raw material sample by its heating at a required temperature for a specified period of time. The exsiccation of samples is performed in special packages of loosely glued duplicator or newsprint paper.

A precisely weighed 4–6-g portion of dry lactulose was placed into a paper package, which was dried and weighed on an analytical balance with an accuracy of up to 0.01 g, and uniformly spread over the entire surface of the package. Paper filters of 11–12.5 mm in diameter were used to manufacture the packages.

The package with its content was weighed and placed between the plates of the drier. Drying at a temperature of 80 ± 0.5°C lasted for 5 min. The dried package with lactulose was cooled to room temperature in a desiccator and then weighed.

The moisture content in dry lactulose was determined by the formula (1):

$$W = \frac{M_1 - M_2}{M} \times 100, \quad (1)$$

where M_1 is the mass of the package with a lactulose portion before drying, g, M_2 is the mass of the package with a lactulose portion after drying, g, and M is the mass of a lactulose portion taken for drying, g.

The hygroscopicity of dry lactulose powders was determined using a desiccator with 3 cm^3 of water poured on its bottom.

The precisely weighed portion of lactulose in a weighting bottle was placed into a desiccator. No more than 6 weighting bottles were uniformly arranged over the entire surface of the desiccator insertion. The weighting bottles placed into the desiccator were opened, and their stoppers were placed near them. The desiccator was closed with a cover.

The weighting bottles were allowed to stand in the desiccator for 20 h at a temperature of 25 ± 2°C. Then they were closed with stoppers, taken out of the desiccator, and weighed.

The hygroscopicity of dry lactulose was determined by the formula (2)

$$X = \frac{M_1 - M_2}{M} \times 100, \quad (2)$$

where M_1 is the mass of the weighing bottle with a lactulose portion after moistening, g, M_2 is the mass of the weighing bottle with a lactulose portion before weighing, g, and M is the mass of a lactulose portion, g.

We performed two parallel estimations, the results of which were used to calculate the arithmetic mean with an error of less than 0.1%.

RESULTS AND DISCUSSION

Temperature is a principal factor governing the process of drying. The dependences of the product output on the drying temperature are plotted in Fig. 3. The product output is the quantity of the finished product in percents of the mass fraction of solids in an initial solution.

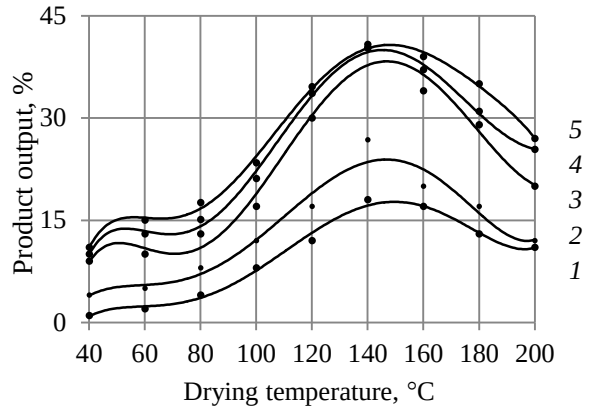


Fig. 3. Product output versus drying temperature at a mass fraction of lactulose of (*1*) 20, (*2*) 30, (*3*) 40, (*4*) 50, and (*5*) 60%.

The analysis of the curves plotted in Fig. 3 shows that the product output grows within a temperature range of 40–140°C. The product output decreases at a heating temperature above 140°C due to such a property of lactulose as the ability to caramelize. At temperatures of 150°C and above, dried crystals adhere to the walls of the drier, thus forming the layer that prevents the accumulation of the finished product.

It has been established that the finished product output increases twice during the drying of solutions with a mass fraction of lactulose of 40–60% in comparison with the drying of solutions with a mass fraction of lactulose of 20 and 30%. However, no appreciable difference between the product outputs in the drying of 40-, 50-, and 60-% solutions has been revealed also due to the effect of caramelization.

Another factor that has an effect on the product output is the solution flow rate. The dependences of the finished product output on the solution flow rate are plotted in Fig. 4.

The dependences plotted in Fig. 4 show that the finished product output grows with an increase in the solution flow rate up to 5–7 ml/min. The flow rate of 1–3 ml/min is optimal for solutions with a mass fraction of lactulose of 20 and 30%, but the product output in this case is only 21 ± 2 and 27 ± 2%, respectively, being twice lower than for solutions with a mass fraction of lactulose of 40–60%. The optimal flow rate for them is 5–7 ml/min. The higher is the solution flow rate, the less is the time spent on the process of drying, so the solution flow rate of 5–7 ml/min and the mass fraction of lactulose in a solution of 40–60% are an optimal proportion.

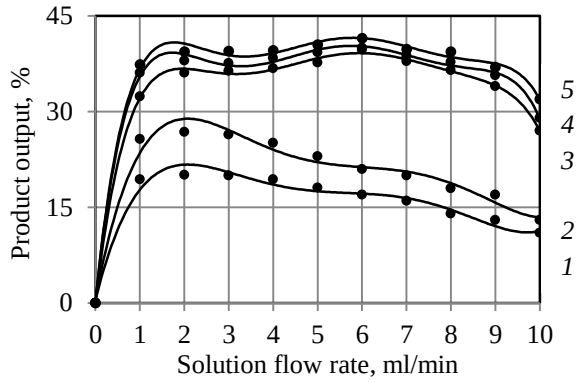


Fig. 4. Product output versus solution flow rate at a mass fraction of lactulose of (*1*) 20, (*2*) 30, (*3*) 40, (*4*) 50, and (*5*) 60%.

Among the principal factors that have an effect on the drying of lactulose is also the air flow rate provided by an aspirator. The aspirator is used to suck air for drying needs in or blow it out through the heater. The dependence of the finished product output on the air flow rate is plotted in Fig. 5.

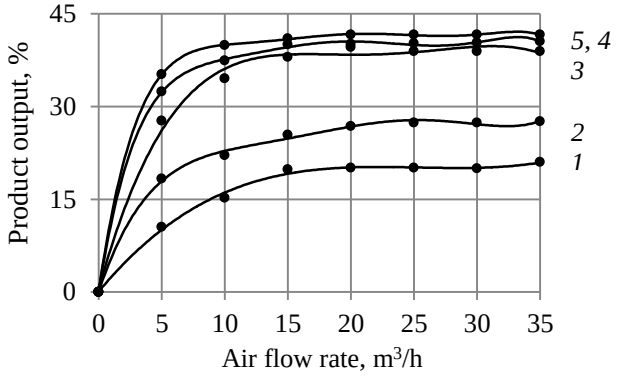


Fig. 5. Product output versus air flow rate at a mass fraction of lactulose of (*1*) 20, (2) 30, (*3*) 40, (*4*) 50, and (*5*) 60%.

The curves plotted in Fig. 5 show the growth of the product output with an increase in the air flow rate up to 15 m^3/h. This is due to the fact that the higher is the air flow rate provided by the aspirator, the faster the particles entrained by this flow will enter the catcher. The air flow rate of no less than 15 m^3/h is optimal for all solutions. The product output considerably grows for the drying of solutions with a mass fraction of lactulose of 40–60% in comparison with 20- and 30-% lactulose solutions (40.3 and 20.1–26.8%, respectively).

Analyzing the data obtained by studying the dependence of the finished product output on the principal drying parameters, we have established that the maximum productivity of the setup is provided for the drying of solutions with a mass fraction of lactulose of 40–60%.

The finished product output and the process time are important factors that govern the drying productivity (Table 3).

Table 3. Output and average duration for the drying of solutions with different mass fraction of lactulose

Mass fraction of lactulose in a solution, %	Maximum product output (theoretical), %	Experimental product output (in % of theoretical)	Specific drying time, ml/min
20	24.8	20.1	0.45 ± 5
30	37.2	26.8	0.55 ± 5
40	49.6	40.3	0.65 ± 5
50	62.0	40.3	0.75 ± 5
60	74.4	40.3	0.85 ± 5

The data given in Table 3 show the growth of the finished product output with an increase in the mass fraction of lactulose in solutions, and the drying time also grows.

The analysis of the data given in Table 3 shows that it is more reasonable to dry a 40-% lactulose solution, as the process time is minimal and the output of crystalline lactulose is maximal.

The quality of the finished product was estimated by such parameters as particle size, moisture content, solubility index, and hygroscopicity.

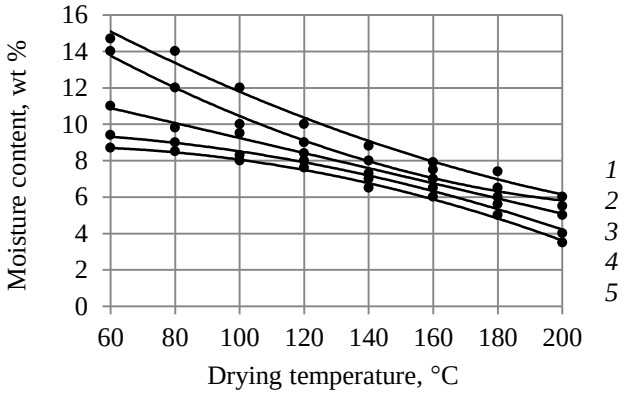


Fig. 6. Moisture content versus drying temperature at a mass fraction of lactulose of (*1*) 20, (*2*) 30, (*3*) 40, (*4*) 50, and (*5*) 60%.

The change of the moisture content in lactulose depending on the solution drying temperature is illustrated in Fig. 6.

The analysis of the curves plotted in Fig. 6 allows us to conclude that the moisture content in the finished product decreases with an increase in the drying temperature and the mass fraction of lactulose in a solution.

The analysis of literature and patent sources shows that the optimal moisture content in crystalline lactulose is less than 7%. Such moisture content values were obtained for the drying of all solutions at a temperature of 120°C and higher.

However, the air flow rate also has an effect on the moisture content in addition to the drying temperature. This is explained by the fact that the drying efficiency grows with increasing air flow intensity, thus reducing the residual moisture in a product and, consequently, the moisture content (Fig. 7).

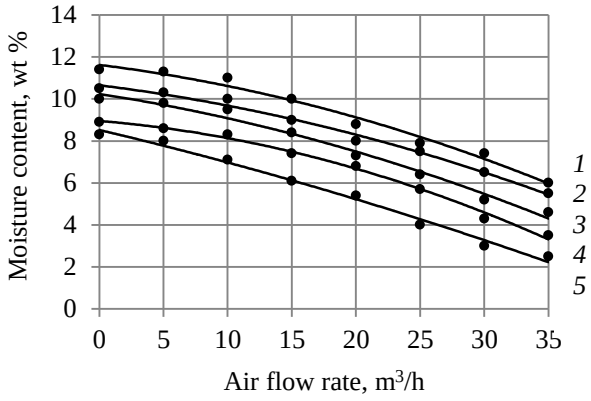


Fig. 7. Moisture content versus air flow rate at a mass fraction of lactulose of (*1*) 20, (*2*) 30, (*3*) 40, (*4*) 50, (*5*) 60%.

From the analysis of the curves plotted in Fig. 7 it can be established that the moisture content in the finished product decreases with an increase in the air flow rate and the mass fraction of lactulose. The optimal air flow rate for the standard moisture content that must not exceed 7% and solutions with a mass fraction of lactulose of 40–60% is no less than 35 m^3/h. The standard parameters for the drying of 20–30-% solutions can be attained only at air flow rates of higher than 25 m^3/h. However, according to Fig. 5, the drying of solutions with mass fractions of 40–60% is most reasonable.

Another factor that has an effect on the moisture content in the finished product is the solution flow rate, as the partial pressure of water vapor also grows with an increase in this parameter. The change of the moisture content in the finished product depending on the solution flow rate is illustrated in Fig. 8.

As shown by the curves plotted in Fig. 8, the moisture content in lactulose grows with an increase in the flow rate of the solution fed to the setup. At required moisture content values of no more than 7%, the flow rate is 1.5–3 ml/min for solutions with a mass fraction of lactulose of 20–30% and 4.5–7 ml/min for 40–60-% solutions. Relying on the earlier obtained data, we can conclude that the product output grows with increasing solution flow rate and, consequently, it is reasonable to dry solutions with a mass fraction of 40–60%.

The following parameter that has an effect on the quality of the finished product is the particle size, as the solubility index and hygroscopicity of dry lactulose directly depend on this parameter.

The dependences of the particle size on the solution flow rate are plotted in Fig. 9.

The curves plotted in Fig. 9 show that the particle size of the finished product grows with an increase in the solution flow rate and the mass fraction of lactulose in a solution. In compliance with the required particle size of 7–10 μm, the optimal flow rate is 5–10, 1–5, and 1 ml/min for a solution with a mass fraction of lactulose of 40, 50, and 60%, respectively. The maximum particle size of the finished product for the drying of solutions with a mass fraction of 20–30% is up to 5 μm.

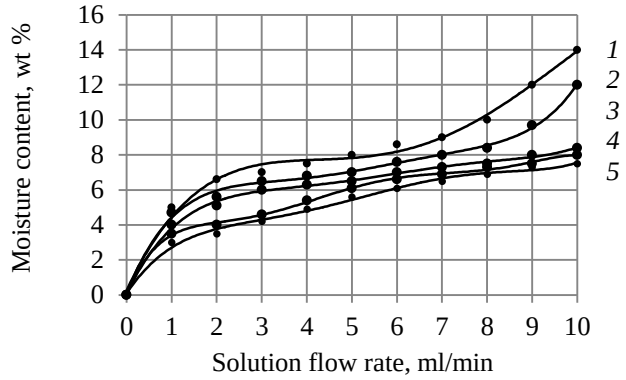


Fig. 8. Moisture content versus flow rate at a mass fraction of lactulose of (*1*) 20, (*2*) 30, (*3*) 40, (*4*) 50, and (*5*) 60%.

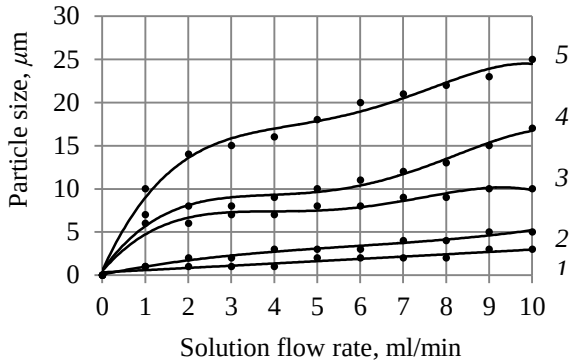


Fig. 9. Particle size versus solution flow rate at a mass fraction of lactulose of (*1*) 20, (*2*) 30, (*3*) 40, (*4*) 50, and (*5*) 60%.

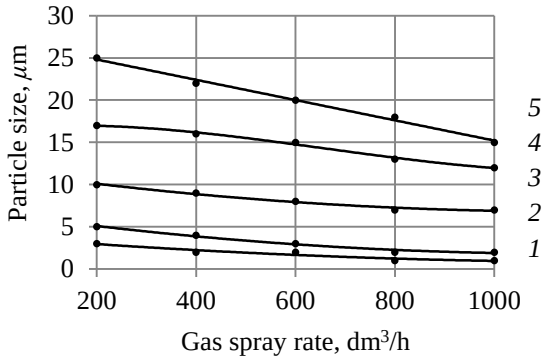


Fig. 10. Particle size versus gas spray rate at a mass fraction of lactulose of (*1*) 20, (*2*) 30, (*3*) 40, (*4*) 50, (*5*) 60%.

Alongside with the solution flow rate, the gas spray rate has a great effect on the particle size. When this parameter is increased, sprayed liquid drops become smaller and, correspondingly, the particle size of the finished product is also reduced (Fig. 10).

According to the curves plotted in Fig. 10, the particle size becomes smaller with increasing gas spray rate. Knowing that the required particle size is 7–10 μm, we have established the optimal gas spray rate range of 500–700 dm^3/h for a solution with a mass fraction of lactulose 40%, as the particle size of the finished product for the drying of solutions with a mass fraction of lactulose of 20, 30, 50, and 60% does not meet requirements. The drying of a 40-% lactulose solution at a specified gas spray rate allows us to obtain a product with a particle size corresponding to the solubility indices (Table 4).

Table 4. Solubility index and hygroscopicity of lactulose with different particle sizes

Mass fraction of lactulose in a solution, %	Particle size, μm	Solubility index, cm^3 of moist precipitate	Hygroscopicity, %
20	1–2	0.10 ± 0.05	25 ± 5
30	3–5	0.15 ± 0.05	20 ± 5
40	7–10	0.20 ± 0.05	10 ± 5
50	11–15	0.30 ± 0.05	5 ± 5
60	16–25	0.30 ± 0.05	1 ± 5

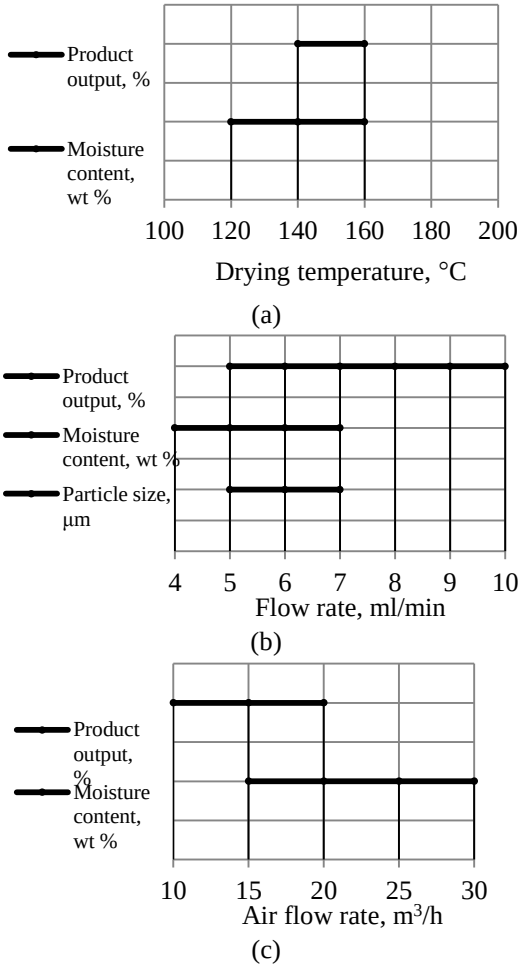


Fig. 11. Optimal parameters for solutions with a lactulose mass fraction of 40%: (a) temperature, (b) flow rate, (c) air flow rate.

The data of Table 4 show that the solubility index grows with increasing particle size and, consequently, the solubility of a product with a greater particle size becomes worse. The obtained lactulose meet the requirements to nutrient carbohydrates, whose solubility index must not exceed 0.4–0.5 cm^3 of moist precipitate. The hygroscopicity of a product decreases with an increase in the mass fraction of lactulose in a solution.

With the data obtained from the analysis of the curves shown in Figs. 6–10, it becomes clear that the drying of solutions with a mass fraction of lactulose of

40% is most rational, as confirmed by the results of studying the finished product output (Figs. 3–5).

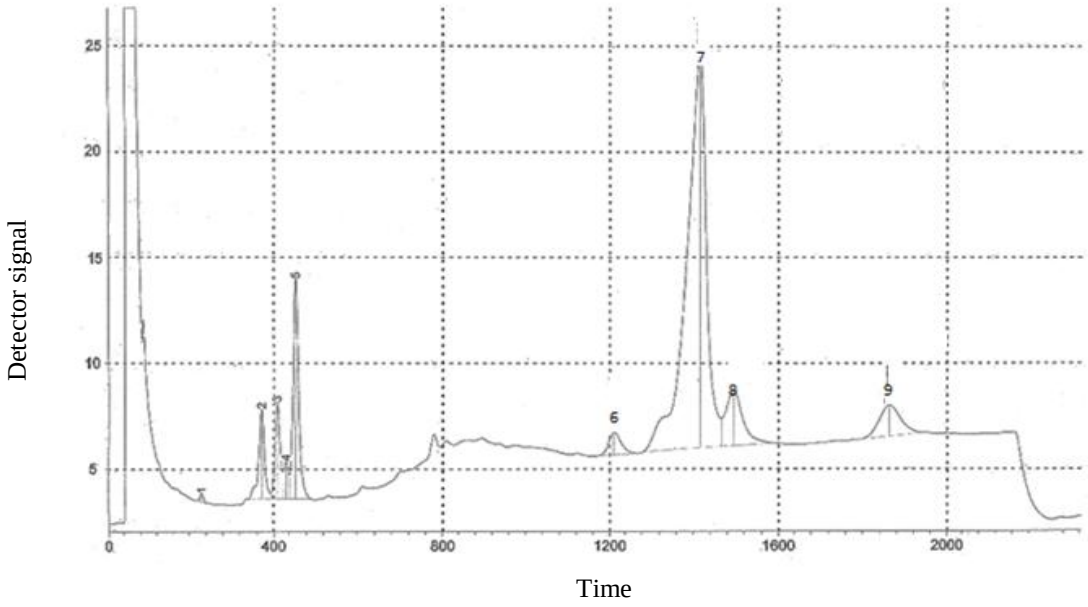
Analyzing the above considered data on the principal factors responsible for the process of drying, let us construct the summary plot of the optimal parameters for the selected solution with a mass fraction of lactulose of 40%. When specifying the drying temperature, we took into account the moisture content in the finished product and its output (Fig. 11).

To determine the solution flow rate values, we considered such parameters as the moisture content in the finished product and its output and particle size (Fig. 11b). It has been established that the flow rate of 5–7 ml/min is optimal.

The optimal air flow rates are plotted in Fig. 11c. The optimal range of air flow rates of 15–25 m^3/h has been established.According to the data plotted in Fig. 11a, the finished product output is maximal at a drying temperature of 140–160°C, and the moisture content meets the requirements at 120–160°C. Hence, the temperatures of 140–160°C are optimal. To confirm the results of studying the qualitative characteristics of dry lactulose, the obtained sample and the initial lactulose solution were subjected to chromatographic analysis. The GLC method has been selected for this purpose, as it has high precision and resolution and allows the quantitative and qualitative analysis for lactulose in the presence of α- and β-lactulose, galactose, glucose, tagatose, fructose, and other carbohydrates (Figs. 12 and 13). The abscissa is the chromatographic run time (mobile phase volume), and the ordinate is the analytical signal, which depends on the concentration of components in the eluent (response).

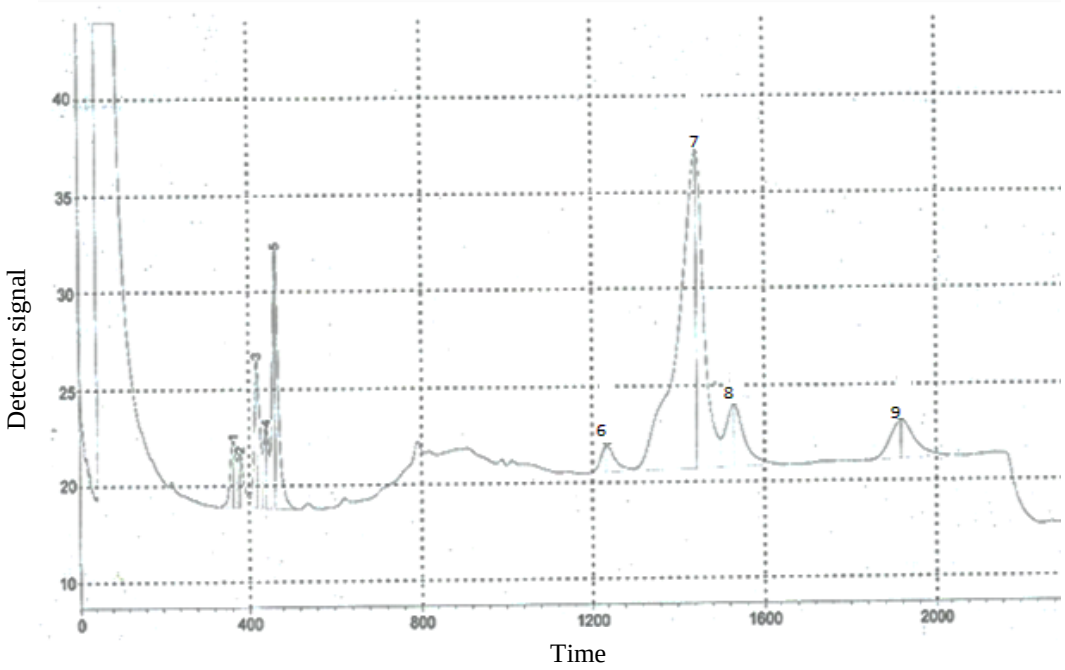
From the data shown in Figs. 12 and 13 it can be established that the concentration of lactulose is 40.4% in the initial syrup and 64.2% in the finished product. Hence, we have managed to increase the concentration of lactulose by more than 20% using the method of spray drying.

Among the advantages of dry lactulose are precise dosing, compactness, packing and transportation convenience, prolonged storage, and the possibility of its target application in the form of a solution. Dry lactulose forms have a considerable advantage in medicine: the preparation is well-digestible due to its large active area of contact in the gastrointestinal tract.



No	Name	Time	Height	Area	Concentration	Dose
1		360	3.5	56.3	8.9	1.0
2		375	2.8	46.8	7.4	1.0
3		415	7.7	117.6	6.0	1.0
4		435	4.2	58.8	3.0	1.0
5		457	13.4	214.6	10.9	1.0
6	ε-lactose	1221	1.4	52.2	2.6	1.0
7	lactulose	1430	16.6	1126.7	40.4	1.0
8	α-lactose	1514	3.3	164.8	11.4	1.0
9	β-lactose	1899	2.0	133.4	6.8	1.0
In sum			54.7	1971.3	100.0	

Fig. 12. GLC pattern of a lactulose solution with a mass fraction of solids of 40%.



No	Name	Time	Height	Area	Concentration	Dose
1		226	0.4	4.0	0.3	1.0
2		368	4.3	70.8	4.5	1.0
3		405	4.6	66.0	4.2	1.0
4		425	1.8	23.0	1.5	1.0
5		448	10.4	159.1	10.1	1.0
6	*ε*-lactose	1198	1.1	36.8	2.3	1.0
7	lactulose	1401	18.6	1015.1	64.2	1.0
8	*α*-lactose	1479	2.6	122.1	7.7	1.0
9	*β*-lactose	1844	1.5	85.4	5.4	1.0
In sum			45.2	1582.3	100.0	

Fig. 13. GLC pattern of dry lactulose.

REFERENCES

1. Gavrilov, G.B., *Tekhnologiya membrannykh protsessov pererabotki molochnoi syvorotki i sozdanie produktov s funktsional'nymi svoistvami* (Technology of Membrane Whey Processing and Creation of Products with Functional Properties), Moscow: Rossel'khozakademiya, 2006.
2. Voskuijl, W, de Lorijn, F, Verwijs, W, et al., PEG 3350 (Transipeg) versus lactulose in the treatment of childhood functional constipation: a double blind, randomised, controlled, multicentre trial, *Gut*, 2004, vol. 53, no. 11, pp. 1590–1594.
3. Petuely, F., Bifidusflora bei Flaschenkindern durch bifidogene Substanzen (Bifidusfactor), *Z. Kinderheimkd*, 1957, vol. 79, no. 2, pp.174–179.
4. Shukla, S., Shukla, A., Mehboob, S., and Guha, S., Meta-analysis: the effects of gut flora modulation using prebiotics, probiotics and synbiotics on minimal hepatic encephalopathy, *Alimentary Pharmacology & Therapeutics*, 2011, vol. 33, no. 6, pp. 662–671.
5. Khramtsov, A.G., Brykalov, B.A., and Kharitonov, D.V., *Lactuloza: tsennost', ispol'zovanie, marketing i effektivnost' proizvodstva* (Lactulose: Value, Application, Marketing, and Production Efficiency), Stavropol: Argus, 2004.
6. US Patent 41 422 916, 1979.
7. Jpn. Patent 52-21 063, 1972.
8. FRG Patent 2 224 680, 1972.
9. US Patent 3 716 408, 1971.
10. Jpn. Patent 56-39 200, 1981.
11. US Patent 4 605 646, 1986.
12. US Patent 5 644 050, 1993.
13. US Patent 5 415 695, 1988.
14. US Patent 5 480 491, 1993.
15. US Patent 5 688 521, 1994.

DEVELOPING FERMENTED GOAT MILK CONTAINING PROBIOTIC BACTERIA

Chuluunbat Tsend-Ayusha* and Yoh-Chang Yoon**

*School of Food Engineering and Biotechnology,
the Mongolian University of Science and Technology (MUST),
Ulaanbaatar, Mongolia, e-mail: tsend@must.edu.mn; chu_tsend@yahoo.com

** Department of Food Science and Biotechnology of Animal Resources,
College of Animal Bioscience and Technology, Konkuk University,
Seoul, Korea

Received September 15, 2013; accepted in revised form November 29, 2013

Abstract: The aim of this study was to develop probiotic fermented goat milk. Goat milk was inoculated with single (*Bifidobacterium longum* and *Lactobacillus acidophilus*) and mixed cultures containing *Bifidobacterium longum* and *Lactobacillus acidophilus*. Goat milk was pasteurized at 95°C for 5 min, cooled to 37±1°C, and inoculated with the required culture before incubation. The combined culture *B. longum* : *L. acidophilus* (8 : 1) was used when it had attained a pH 4.5±2 and a titratable acidity 65–70. Goat milk for fermented milk was sterilized at 120°C for 10 min, cooled to 37°C, and inoculated with 8–10% of the bifidobacterium culture, and goat milk was pasteurized at 95°C for 20 min, cooled to 42°C, and inoculated with 3% of the L. acidophilus culture. The cultures were used when they attained pH 4.3 and 4.5 and titratable acidity between 56 and 800T. The developed technology of fermented milks and the characteristics of fermented goat milk were studied. According to the findings, we concluded that fermented goat milk was balanced by the amino acid content and products of high biological value. Fermented goat milk with the expected beneficial health effect is based on a high concentration of probiotic bacteria.

Key words: probiotics, *Bifidobacterium longum, Lactobacillus acidophilus*, amino acids, biological value

UDC 637.12'6.04/.07
DOI 10.12737/2050

INTRODUCTION

Fermented milk enriched with probiotic bacteria has developed into a very successful category of functional foods.

In recent years, interest has increasingly focused on foods that affect health positively beyond their nutritional value. Among these functional foods, much attention has been given to probiotic products. Probiotic foods contain microorganisms or components of microbial cells that have a beneficial effect on the health and well-being of the consumer host [5]. The viability of probiotic bacteria of high counts (at least 10^7 cfu/g, or cfu/mL, of product) is recognized as an important requirement during the manufacturing and marketing of probiotic foods in order to achieve the claimed health benefits.

Goat milk has been described as having a higher digestibility and lower allergenic properties than cow milk. In addition, goat milk has been attributed with certain therapeutic values in human nutrition [1].

According to statistical data, the livestock population in Mongolia increased to 42 million head. The goat population is increasing especially due to the growing price of cashmere in the world market. Therefore, the goat milk recourse is increasing in Mongolia.

In order to organize the industrial processing of goat milk and the production of dairy products in Mongolia, we have studied the chemical composition of the goat milk of the Mongolian breed, especially to identify amino acids, minerals, and vitamins, as well as the fractional structure of whey proteins in this goat milk. We also determined toxic elements and radio nucleoids in goat milk to assess safety. Moreover, we studied the technological properties of goat milk.

The objective of this study was to evaluate the effect of the starter cultures (*Bifidobacterium longum* and *Lactobacillus acidophilus*) during the manufacture of fermented goat milk, grown separately as single species and in combination with bacteria for probiotic production. Then the characteristics of products manufactured from fermented goat milk were studied.

MATERIALS AND METHODS

The object of the study was goat milk from private farms. The starter cultures, *Bifidobacterium longum* and *Lactobacillus acidophilus*, were obtained from Chr. Hansen Laboratories (Copenhagen, Denmark) and were used to develop the technology of fermented milks.

Chemical analysis. The pH value was measured using an pH meter. Total acidity percentage (as lactic acid), fat, protein, lactose, and total solids were determined according to AOAC (2000).

Total bacterial counts were determined by using plate count agar according to the Standard Methods for the Examination of Dairy Products [2, Case et al.].

The amino acid composition was determined using the automatic amino acid analyzer LG-5000 (Germany), based on ion-exchange chromatography. For the estimation of protein quality, the irreplaceable amino acid

score was evaluated using the following formula [FAO/WHO].

RESULTS AND DISCUSSION

At present, probiotic products manufactured from *B. longum* and *L. acidophilus* are practiced widely.

The combined culture was prepared from *Bifidobacterium longum* and *Lactobacillus acidophilus* in a particular proportion (2:1; 5:1; 8:1). The characteristics of the combined cultures were studied. Results are shown in Table 1.

Table 1. Selection the optimal proportion of culture in the combined starter culture

Rate of culture	Incubation time, h	Acidity, ^{0}T	pH	Viability bacterial count (log cfu/g)	
				Bifidobacteria (*B. longum*)	*L. acidophilus*
2 : 1	4.5	73–78	4.64	7 x 10^7	4 x 10^8
5 : 1	6.0	70–75	4.72	5 x 10^8	3 x 10^8
8 : 1	7.5	60–65	4.80	3 x 10^9	2 x 10^8

According to the data in the table, the duration of fermentation for different cultures was 4.5–7.5 hours.

The findings show that the viable cell count of the combined culture was quite high, 2 x 10^8 cfu/g.

Therefore, the 8 : 1 ratio of the cultures was selected to develop a fermented milk technology. Probiotic fermented milk was prepared from fresh goat milk.

The development of *B. longum*, *L. acidophilus*, and *B. longum : L. acidophilus* (8 : 1) cultures was inoculated at 10.3 and 4% and was followed by measuring changes in titratable acidity and pH. At the first stage, the prepared fermented combined culture was used.

The goat milk was pasteurized at 95°C for 5 min, cooled to 37±1°C, and inoculated with the required culture before incubation. The combined culture *B. longum : L. acidophilus* (8 : 1) was used when it attained pH 4.5±2 and titratable acidity 65–70.

The next stage of preparing fermented milk used two different starter cultures.

Goat milk for fermented milk was sterilized at 120°C for 10 min, cooled to 37°C, and inoculated with 8–10% of the bifidobacterium culture, and goat milk was pasteurized at 95°C for 20 min, cooled to 42°C, and inoculated with 3% of the *L. acidophilus* culture.

The cultures were used when they attained pH 4.3 and 4.5 and a titratable acidity between 56 and 80^{0}T.

The developed technology of fermented milks and the characteristics of fermented goat milk were studied.

Biological value of fermented milk. The quality of foodstuffs is characterized by their chemical composition, physical properties, and nutrient and biological values. For all this, biological value is the most important parameter as it determines how products correspond to the optimal needs of human physiological norms.

As we all know, biological value reflects the quality of protien components in products and the level of balance in amino acidity composition.

Proteins in some traditional foodstuffs and in foods obtained from new sources differ in the content of irreplaceable amino acids. Therefore, their amino acidity was calculated for evaluating the biological value of fermented milk (Table 4).

Table 2. Physicochemical and microbiological characteristics of fermented goat milk

Parameter	Fermented milk		
	With *L. acidophilus*	With bifido bacteria	With combined culture
Fat, %	3.5	3.5	3.5
Protein, %	3.3	3.3	3.3
Acidity, ^{0}T	80	56	70
Bacterial count, (cfu/g)	10^8	10^9	10^9

Table 3. Free amino acid compositions of goat fermented milk

Amino acids, %	Fermented milk		
	With *L. Acidophilus*	With bifido bacteria	With combined culture
Valine	5.5	6.7	6.9
Isoleucine	3.9	3.8	6.2
Leucine	8.7	8.4	9.1
Lysine	7.3	7.2	7.5
Methionine + Cystine	6.4	6.7	3.7
Tryptophane	1.3	1.6	1.8
Threonine	4.48	4.87	4.9
Phenylalanine + Tyrosine	8.43	8.63	9.4
Alanine	3.85	3.34	3.6
Arginine	7.48	7.44	3.5
Aspartate	7.54	7.55	6.9
Hystidine	2.95	2.95	24.9
Glycine	1.95	3.04	2.1
Glutamate	14.24	13.64	22.0
Proline	5.06	4.91	10.6
Serine	3.97	3.55	5.7

Table 4. Irreplaceable amino acids of fermented goat milk

Amino acids, %	Fermented milk		
	With *L. Acidophilus*	With bifido bacteria	With combined culture
Valine	122.6	108.0	133.7
Leucine	143.6	140.5	124.0
Isoleucine	109.6	104.5	145.6
Lysine	134.2	130.1	131.2
Methionine + Cystine	115.1	110.2	118.9
Threonine	133.5	122.6	133.7
Tryptophane	144.3	156.0	124.0
Phenylalanine + Tyrosine	145.2	137.3	145.6

The biological value of protein is characterized to a considerable extent by the content of irreplaceable amino acids compared to the "ideal" FAO/WHO protein.

The data in the table show that irreplaceable amino acids in fermented milks are well balanced.

CONCLUSIONS

According to the results of the study, we concluded that fermented goat milk products were balanced in their amino acid content and were of a high biological value. The fermented goat milk retains high counts from added probiotics, which are recommended to produce therapeutic effects.

REFERENCES

1. Alférez, M.J., Barrionuevo, M., López-Aliaga, I., Sanz-Sampelayo, M.R., Lisbona, F., Robles, J.C., and Campos, M.S., Digestive utilization of goat and cow milk fat in malabsorption syndrome, *Journal of Dairy Research*, 2001, vol. 68, no. 3, pp. 451–461.
2. *Standard Methods for the Examination of Dairy Products*, Richardson, G.H., Ed., Washington, DC: American Public Health Association, 1985, 15th ed.
3. *Mongolian Statistical Yearbook 2013*, Ulaanbaatar: National Statistics Office of Mongolia, 2013.
4. Tsend-Ayush, Ch. and Ganina, V.I., The micronutrient composition of the milk of Mongolian small pasture cattle, *Khranenie i pererabotka sel'khozsyr'ya (Storage and Processing of Agricultural Raw Materials), 2009, no. 9, pp. 32–34.*
5. Salminen, S., Ouwehand, A., Benno, Y., and Lee, Y. K., Probiotics: How should they be defined? 1999, *Trends in Food Science & Technology*, vol. 10, no. 3, pp. 107–110.

BIOTECHNOLOGY

A METHOD FOR PROCESSING OF KERATIN-CONTAINING RAW MATERIAL USING A KERATINASE-PRODUCING MICROORGANISM STREPTOMYCES ORNATUS S 1220

A. Yu. Poletaev, O. V. Kriger, and P. V. Mitrokhin

Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone/fax: +7 (3842) 39-05-37, e-mail: olgakriger58@mail.ru

Received May 25, 2013; accepted in revised form October 14, 2013

Abstract: The effect of substrate on mycelium growth, the optimal composition of the culture medium, and the optimal cultivation conditions for Streptomyces ornatus S 1220 have been investigated in the present work. The specific activity of keratinase has been monitored during cultivation and activity variation caused by addition of various salts to the cultivation medium has been analyzed. The results of the optimization study are reported and successful use of the culture studied in the present work in processes performed on an industrial scale is anticipated.

Key words: keratin, secondary raw materials, processing, keratinase-producing microorganism, enzyme, protein, cultivation, keratinase activity, bioconversion

UDC 577.151.35:547.962.9
DOI 10.12737/2051

INTRODUCTION

The amount of secondary raw material obtained during slaughter and processing of poultry can be as high as 45% of the live weight of the birds. Most of this material is constituted by down and feathers, which have a high biological value, since they contain about 85% keratin.

Keratins are abundant proteins found in epithelial cells. They are major structural components of skin, nails, hair, feathers, and wool. Analysis of the amino acid composition of keratins shows that these proteins are a rich source of essential amino acids. However, the transformation of natural keratin into a digestible form is problematic, since keratins are fibrillar proteins and their mechanical stability is higher than that of all other materials of biological origin except chitin. The conventional techniques used for the processing of keratin-containing raw materials are neither efficient nor rational. The use of physical and chemical processing methods can result in formation of various toxic substances, as well as in loss of up to 75% of protein. Consequently, novel procedures for the processing and efficient use of the secondary raw material are needed.

Enzymatic methods for the processing of protein-containing raw materials, which became available due to the development of biotechnology, allow for the preservation of all essential amino acids. The use of ready-made enzyme preparations on an industrial scale can lead to a significant increase in costs and expenses, and therefore it is necessary to find solutions which enable the minimization of processing costs for the keratin-containing raw material. The use of live microorganism cultures in the processing of keratin-containing raw material allows for a reduction of the processing costs. The bioconversion method involves cultivation of the enzyme-producing strains on a substrate formed by the raw material to be processed. High efficiency of the subsequent decomposition of the substrate can be attained if this method is used. Selection of the optimal enzyme-producing microorganism and the optimal cultivation conditions is necessary in order to increase the rate and efficiency of bioconversion employing this method.

With the requirements concerning the strain and its functional efficiency taken into account, we chose the keratinase-producing strain Streptomyces ornatus S 1220. Simple composition and low cost of cultivation media, high levels of keratinase production, short cultivation time, and high enzyme yield [4] were the primary reasons for the choice of this strain.

The aim of the present work, formulated with the current problems taken into account, was to define the optimal composition of the cultivation medium providing for a high biomass yield of Streptomyces ornatus S 1220, to determine the most appropriate cultivation temperature and the period during which the rate of biomass accumulation is the highest, and to assess the effect of chemical additives on the specific enzyme activity. The tasks to be fulfilled in order to achieve the aim included analysis of the chemical composition of feathers, characterization of the effect of complex cultivation media on the yield of keratinase produced by the microorganism, analysis of environmental effects on the growth and productivity of the microorganism under investigation, and optimization of the cultivation parameters established.

MATERIALS AND METHODS

The concentration of cations in the feathers was determined using a Kapel'-105 capillary electrophoresis system, a VSL-200 laboratory balance, and an SM-50 centrifuge. Total nitrogen/protein concentration was determined using a Rapid N cube total nitrogen/protein analyzer and a VSL-200 laboratory balance. Protein content in the biomass was determined according to European standards using a RAPID N ELEMENTAR protein analyzer. The method is based on combustion of a pre-weighed portion of the substance under investigation. The combustion performed in the presence of oxygen at a high temperature (approximately 900°C) results in the formation of carbon dioxide, water, and nitrogen; the amount of the latter is measured by the device.

The nutrient media used in the present work included starch-based medium (GOST (State Standard) R 52060-2003), selective Czapek medium (TU (Technical Specifications) 9229-014-00419789-95), and meat-peptone agar (MPA, GOST (State Standard)-17206-96). The effect of chemicals present in the substrate on the increase of the specific keratinase activity was assessed using cultivation of Streptomyces ornatus S 1220 on nutrient broth supplemented with one of the following salts: NaCl, KH_2PO_4, or $CaCO_3$. Keratinase activity was assayed using a modification-based method: namely, 200 mg of ground feathers (rinsed with chloroform and water and air-dried) were mixed with 10 mL of 0.05 M borate buffer, pH 9.0, and 1 mL of culture filtrate, shaken vigorously and incubated at 37 ± 1°C for 3 hours in order for keratin hydrolysis to occur. Two control incubations were started simultaneously in order to assess the solubility of feathers in the buffer and the content of soluble protein in the culture medium. Residual non-cleaved protein was precipitated by trichloroacetic acid (TCA) and separated by filtration. The optical density at the wavelength of 340 nm was measured in the filtrate. The amount of cleaved protein ($\mu g/cm^3$) per 1 h of incubation with the culture medium was determined using a calibration curve constructed using serum albumin solutions.

RESULTS AND DISCUSSION

The total content of protein in the feathers was approximately 87.5% by mass, as determined in the experiments described above. The content of cations in the feathers was determined as well. The results are shown in Table 1.

Investigation of the composition of the feathers confirmed that feathers are a product of high biological value and showed that they contain cations essential for the growth of the streptomycete [4].

Temperature is among the most dramatic effects exerted on a living organism by the environment. A living organism adapts to specific temperature conditions. Temperature determines the growth rate of a microorganism and has an impact on all aspects of its physiological functioning. The growth rate of microorganisms can either decrease or increase as the temperature changes. Since the reported optimal temperature for mycelial growth is about 30°C [3], we cultivated the microorganisms at 30 ± 1 or 37 ± 1°C on one of the three culture media: starch agar, MPA, or Czapek medium. Inoculated plates were placed in a thermostat at temperatures indicated above.

Table 1. Mass concentration of cations in feather

Parameter	Hydrolyzate volume, mL	Cation concentration in the sample, mg/dm^3		Average value, mg/dm^3	Content of the cation in the sample, x±Δ, g/kg
		1	2		
Potassium	12.5	6.861	6.794	6.828	17.07±1.72
Sodium	12.5	16.888	16.675	16.782	41.96±4.22
Magnesium	12.5	1.795	1.775	1.785	4.46±0.45
Calcium	12.5	8.933	8.357	8.645	21.61±0.22

The most suitable medium and temperature for biomass expansion were established by cultivating Streptomyces ornatus S 1220 on media described above for 7 days and measuring the protein content in the biomass. The colonies grown on the plates were counted and the concentration of microorganisms per 1 g biomass was determined. The amount of biomass was maximal after 7 days of cultivation on all three culture media tested, but the rate of biomass accumulation was maximal during the first three days of cultivation and decreased significantly afterwards. The optimal cultivation temperature was 30 ± 1°C for all three culture media tested. The results are shown in Table 2.

Table 2. Biomass accumulation during the cultivation of Streptomyces ornatus S 1220 at different temperatures

Temperature, ºC	Number of microorganisms, $\times 10^{-3}$ CFU/g			
	Day 1	Day 3	Day 5	Day 7
Starch agar				
30.00	146	345	360	375
37.00	16	35	50	52
MPA				
30.00	200	590	630	645
37.00	20	100	105	110
Czapek medium				
30.00	101	235	250	265
37.00	10	35	38	40

Table 2 shows that the keratinase-producing microorganism becomes less active as temperature increases and that biomass accumulation is the most intensive upon cultivation on meat-peptone agar.

In a subsequent experiment, the total protein content was monitored during 7 days. The experiment was carried out at 30°C, since this temperature provided for the most intensive biomass growth. The total protein content was calculated by multiplying the total nitrogen content by a coefficient which is equal 6.25 for living organisms. The results are shown in Table 3.

A relatively intensive protein accumulation was detected in the culture grown on MPA at 30°C, with the protein content amounting to about 70% on the seventh day of cultivation. Therefore, meat-peptone agar was chosen for further studies.

Table 3. Protein accumulation in the biomass during cultivation of Streptomyces ornatus S 1220 at the optimal temperature (30ºC)

Protein content in the mycelium, mass %			
day			
1	3	5	7
Starch agar			
2.04	18	24	26
Nutrient agar			
5.6	59	68	70
Czapek medium			
5.9	22	26	30

After the optimal conditions for the cultivation of Streptomyces ornatus S 1220 were determined, further experiments aimed at the optimization of parameters affecting keratinase activity were performed.

The rate of an enzymatic reaction is affected by multiple factors. Selection of nutrient media components providing for an increase of the specific activity of keratinase is necessary to delineate the best conditions for the conversion of keratin-containing raw materials into amino acids.

The effect of salts (NaCl, KH_2PO_4, and $CaCO_3$) added to the cultivation medium on the specific activity of keratinase was monitored during 7 days. The concentration of the salts was in accordance with the earlier studies and equaled 0.25% for NaCl, 0.1% for KH_2PO_4, and 0.3% for $CaCO_3$ [1, 2]. After cultivation, the culture liquid was separated from the mycelium by centrifugation (3500–4000 rpm), and the supernatant was filtered. Keratinase activity of the filtrate was assayed [5]. The values of specific keratinase activity were registered on the first, third, fifth, and seventh days of cultivation. The results are shown in Table 4.

Table 4. Changes of the specific activity of keratinase during cultivation of Streptomyces ornatus S 1220

Specific activity of keratinase, U/mg protein			
day			
1	3	5	7
NaCl			
3.4	11.2	12.7	13.2
KH_2PO_4			
2.6	11.0	13.2	14.7
$CaCO_3$			
2.5	11.7	12.8	13.5

As shown in Table 4, the specific activity of keratinase increased throughout the 7 days of cultivation in media supplemented with the salts named above, but the highest value of the specific activity of keratinase was achieved after 7 days of cultivation in the presence of KH_2PO_4. The activity increased significantly in all samples after three days of cultivation, however, the activity increase was most pronounced in the culture supplemented with calcium carbonate (11.7 U/mg).

Besides, titratable and active acidity and specific keratinase activity were monitored during seven days of cultivation at 30°C in a medium not supplemented with any salts. The results are shown in Table 5. No changes in active acidity were detected; it remained at the level of 6.5 units during the 7 days of cultivation. Titratable acidity decreased from 18 to 16°T during the time period from day 3 to day 7, this being indicative of a decrease of the total amount of acids in the culture medium towards the end of the incubation. At the same time, the specific activity of keratinase increased significantly during the first three days and continued to increase slightly until the end of cultivation. This implies a connection between the increase of keratinase concentration and the high rate of biomass accumulation during the first three days of cultivation.

Table 5. Changes of pH, T, and keratinase activity during the cultivation of Streptomyces ornatus S 1220 on MPB at 30°C

Parameter	Day			
	1	3	5	7
Titratable acidity, °T	18	18	17	16
Active acidity, pH units	6.5	6.5	6.5	6.5
Specific keratinase activity, U/mg protein	2.6	11.0	11.9	12.8

CONCLUSION

The studies performed showed that the optimal duration of the cultivation of Streptomyces ornatus S 1220 is three days; the microorganism must be cultivated at 30°C on MPB supplemented with $CaCO_3$, since addition of the salt results in an increase of keratinase activity. The optimal duration of streptomycete cultivation is three days, since the rate of biomass accumulation is the highest during this time. Moreover, the activity of keratinase in the culture medium attains a value sufficient for efficient conversion of keratin-containing raw material after three days of cultivation.

In our opinion, implementation of the parameters defined in the present study will allow for efficient use of the Streptomyces ornatus S 1220 strain and increased yield of non-essential and essential amino acids during the processing of keratin derived from feathers. The use of this microbiological procedure for the processing of keratin-containing raw materials will allow for a decrease of the amount of waste in poultry-processing industry and provide a source of amino acids for feedstock, food, and pharmaceutical industry.

REFERENCES

1. Jayalakshmi, T., Krishnamoorthy, P., Ramesh, G., and Sivamani, P., Isolation and screening of a feather-degrading keratinolytic actinomycetes from Actinomyces sp., *Journal of American Science*, 2010, no. 6, pp. 45–48.
2. Tatineni, R., Doddapaneni, K.K., Potumarthi, R.C., and Mangamoori, L.N., Optimization of keratinase production and enzyme activity using response surface methodology with streptomyces sp7, *Applied Biochemistry and Biotechnology*, 2006, vol. 141, no. 2–3, pp. 187–201.

3. Takiuchi, I., Higichi, D., Sei, Y., and Koga, M., Isolation of an extracellular proteinase (keratinase) from Microsporum canis, *Sabouraudia*, 1982, vol. 20, pp. 281--288.
4. Bandoyan, A.K., Dorokhov, V.V., Noskova, L.B., and Morozova, M.A., RF Patent 2 034 924, 1995.
5. http:// www.claw.ru/a-natural/25453.htm.

HISTIDINE BIOTRANSFORMATION MEDIATED BY L-HISTIDINE-AMMONIA-LYASE

G. V. Borisova and O. V. Bessonova

Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone/fax: +7 (3842) 39-68-45, e-mail: mks.ooo@rambler.ru

Received May 23, 2013; accepted in revised form September 28, 2013

Abstract: Kinetics of the metabolism of the heterocyclic amino acid histidine exposed to the L-histidine ammonia-lyase enzyme has been investigated and the technology of extraction of histidine biotransformation products (urocanic acid and ammonia) from casein hydrolyzates enabling the subsequent use of these hydrolyzates as a milk protein concentrate for the production of specialized dietary products for the nutrition of histidinemia patients has been developed.

Key words: histidine, biotransformation, L-histidine-ammonia-lyase, the removal of ammonia, urocanic acid, histidinemia

UDC 637.147.2:547.784.2
DOI 10.12737/2052

INTRODUCTION

Over 600 inherited metabolic disorders, including hereditary disorders of amino acid metabolism (histidinemia, phenylketonuria, tyrosinemia, alkaptonuria, and others), have been identified to date. Diseases associated with disturbed amino acid metabolism develop if the activity of an enzyme involved in the metabolism of ingested amino acids is selectively reduced. These diseases affect the central nervous system, resulting in mental retardation with motor and speech defects, impaired vision and hearing, emotional and behavioral disorders, and seizures [1, 4].

Hereditary metabolic disorders are caused by mutations introducing changes into the nucleotide sequence of genomic DNA. Mutations result in the synthesis of abnormal proteins (including structural proteins, enzymes, hormones, growth factors, and receptor proteins). For instance, if the mutation affects a gene encoding an enzyme, the latter loses its catalytic activity partially or completely [2, 21]. The vast majority of hereditary metabolic disorders is caused by genetic defects affecting enzymes involved in the metabolism of amino acids, carbohydrates, and lipids. Pathogenesis and clinical manifestations are determined by the lack of normal metabolites (intermediate or final) and the accumulation of toxic metabolites. The clinical presentation largely depends on the degree of the mutant gene expression, as well as on other genetic factors and environmental conditions. Mental retardation and neurological disorders are among the most frequent and severe consequences of the biochemical defect in the majority of patients [3, 5, 17].

Total screening for histidinemia, an innate defect of amino acid metabolism, in newborn babies has been recently introduced in Russia. Histidinemia is a hereditary disease associated with a disturbance of the metabolism of the amino acid histidine. The disease is a congenital defect of histidine-ammonia-lyase, an enzyme that belongs to the lyase class (EC 4.3.1.3, gene HAL, 12q22-q23) and catalyzes the deamination of L-histidine to form urocanic acid and ammonia. Histidine (L-α-amino-β-imidazolylpropionic acid) is an essential amino acid which must be present in the diet of young children. Besides, a histidine residue is often present in the active centers of enzyme molecules, and the biosynthesis of histamine in the human organism requires histidine as well. The metabolic block leads to accumulation of large amounts of histidine and the products of its abnormal metabolism (imidazolepyruvic, imidazolelactic, and imidazoleacetic acids) in the tissues and body fluids of the patient, this having a toxic effect on the central nervous system [8, 9, 14, 19, 24].

Diet therapy based on limiting the ingestion of histidine to a value that is the most appropriate for the individual metabolic requirements of the patient's organism is the only efficient treatment for histidinemia currently available [6, 7, 13, 20].

The aim of the present work was to study histidine biotransformation in milk protein hydrolyzates treated by L-histidine-ammonia-lyase, to develop a technique for biotransformation, and to elaborate a procedure for the production of a milk protein concentrate for use in specialized dietary products.

OBJECTS AND METHODS OF THE STUDY

Casein hydrolyzate obtained by treating milk proteins with an enzyme system including exo- and endopeptidases thermolysin, carboxypeptidase A, and leucine aminopeptidase for 8 ± 0.05 h at a temperature of 50 ± 1°C and the enzyme-substrate ratio of 1:50 was used in the present work. Other materials included L-histidine-ammonia-lyase (8.6 U/mg protein) from Sigma, trisodium citrate 5.5-hydrate approved for food use according to GOST (State Standard) 31227-2004, potable water conforming to GOST (State Standard) 2874, and other chemicals manufactured in Russia and abroad, of a purity not lower than "chemically pure" grade.

Sampling and preparation of the samples for analysis were carried out in accordance with the GOST (State Standard) 26809 "Milk and dairy products. Acceptance procedures, sampling methods, and preparation of samples for analysis". Histidine biotransformation was carried out using a static procedure in a thermostat (temperature 30 ± 2°C) equipped with a stirrer at pH 9 ± 0.01 (phosphate buffer). These values of pH and temperature are optimal for L-histidine ammonia-lyase used in the present work according to earlier reports [8, 16] and information provided by the manufacturer. The duration of biotransformation ranged from 2 to 8 hours; the ratio of enzyme concentration to the concentration of the protein substrate was 1:25, 1:50, or 1:100. The process was terminated by heat inactivation of the enzyme in the hydrolyzate (90–95°C for 5–10 min).

Concentrations of ammonia and urocanic acid were determined using a KAPEL 105 capillary electrophoresis system according to a procedure developed in the scientific and educational center of Kemerovo Institute of Food Science and Technology.

A rotary evaporator IR-1LT intended for rapid removal of solvents from solutions or suspensions of organic and inorganic compounds by film evaporation under normal or reduced pressure and controlled temperature [10] was used to eliminate ammonia from the reaction mixture.

RESULTS AND DISCUSSION

The kinetics of histidine biotransformation in protein hydrolyzates and the effects of various physical and chemical factors on the process rate have been investigated in the present study.

The dependence of the rate of the biotransformation reaction on histidine concentration is shown in Fig. 1.

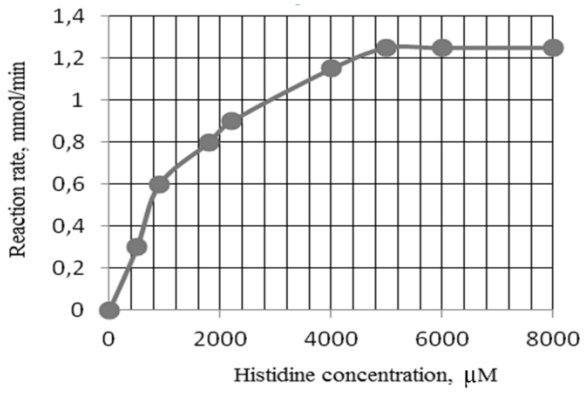


Fig. 1. The dependence of the reaction rate on histidine concentration.

Analysis of the data shown in Fig. 1 allowed for the conclusion that the dependence of the rate of the biotransformation reaction on substrate concentration corresponds to a classical hyperbolic curve. The character of the dependence was investigated at substrate concentrations ranging from 300 to 9000 mM. As shown in the graph, the reaction rate is proportional to the substrate concentration at low substrate concentrations. However, the maximal value of the reaction rate is attained at a histidine concentration of 5000 mM. The enzyme is obviously saturated by the substrate at this concentration, and therefore the concentration of the enzyme-substrate complex ES becomes equal to the enzyme concentration. The hydrolysis rate remains constant at higher substrate concentrations, this apparently being due to substrate inhibition.

The data obtained were analyzed using the Lineweaver-Burke method for the linearization of Michaelis-Menten equation. The results are shown in Fig. 2. The method of least squares was used to determine the tangent of the inclination angle numerically equal to $1/V_m$. The coordinate of the intersection of the extrapolated line with the y-axis corresponds to the K_M/V_m value, and that of the point of intersection with the x-axis corresponds to the K_M value.

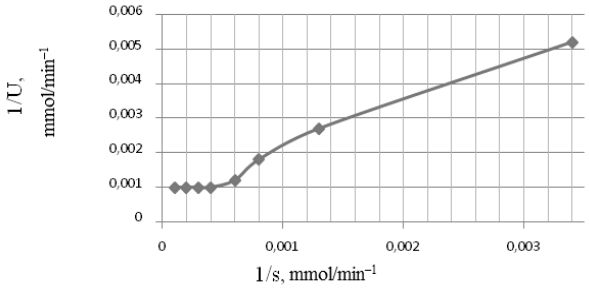
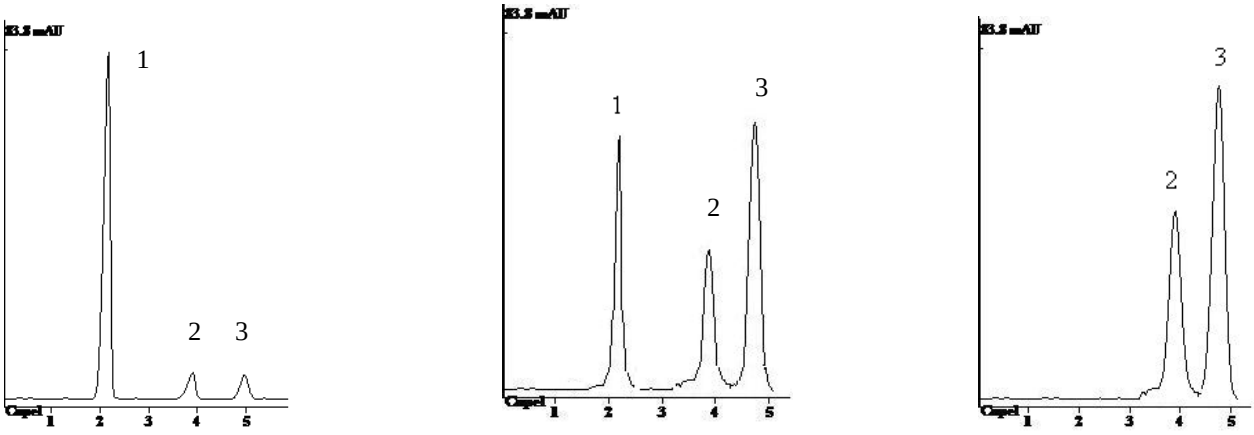


Fig. 2. Graphical determination of the maximal rate of the enzymatic reaction and the Michaelis constant using the Lineweaver-Burke method.

The value of the Michaelis constant determined in the present study equaled 1745 µM, and the maximal rate of the biotransformation process was 1.25 µmol/min. Similar values (K_M = 1751 µM, V_m = 1.15) were reported by other researchers who analyzed the effects of L-histidine-ammonia-lyase under model conditions. It is known that K_M may vary to a certain extent, depending on the ratio of histidine isoforms, buffer composition, and the presence of substances regulating enzyme activity in the reaction medium [11, 15].

The accumulation of the reaction products in the course of histidine biotransformation by L-histidine-ammonia-lyase at the previously determined reaction parameters (K_M = 1751 µM, V_m = 1.15) was analyzed in the subsequent experiments. The optimal conditions for the functioning of L-histidine ammonia-lyase are a temperature of 30 ± 1°C and pH 8.5 [15, 22, 23]. Therefore, the dynamics of ammonia and urocanic acid accumulation have been analyzed for a reaction carried out in tetraborate buffer, pH = 9.2 (λ = 254 nm, voltage 25 kV). The results of the experiment are presented in Fig. 3.



a) b) c)

Fig. 3. Dynamics of ammonia and urocanic acid accumulation during histidine biotransformation mediated by L- histidine-ammonia-lyase, after: a) 2 ± 0.05 h, b) 4 ± 0.05 h, c) 8 ± 0.05 h; 1– histidine; 2–ammonia, 3–urocanic acid.

Analysis of the data presented in Fig. 3 allows for the conclusion that the biotransformation results in intense accumulation of ammonia and urocanic acid, indicative of histidine conversion into the reaction products mediated by L-histidine-ammonia-lyase [18]. Thus, the content of histidine dropped to 71.3 % of the initial value after 2 ± 0.05 h of treatment, to 32.06 % of the initial value after 4 ± 0.05 h, and reached the minimal value at 8 ± 0.05 h. Amino acid composition of the hydrolyzates obtained is shown in Table 1.

Table 1. Amino acid composition of casein hydrolyzates obtained as a result of biotransformation by L- histidine-ammonia-lyase

Amino acids g/100 g	Casein*	Hydrolyzate
Essential, total, including:	40.32	25.29
valine	5.36	0
isoleucine	6.15	4.8
leucine	11.66	10.61
lysine	8.9	4.69
methionine	3.3	3.39
threonine	4.27	1.8
tryptophan	0.68	0
phenylalanine	5.8	0
Non-essential, total, including:	59.68	33.75
alanine	5.39	3.7
arginine	4.45	3.42
aspartic acid	3.33	1.62
histidine	3.33	2.8
glycine	2.42	0.92
glutamic acid	14.59	7.83
proline	12.97	4.2
serine	3.84	1.02
tyrosine	7.56	7.04
cysteine	1.8	1.2
Total	100	59.04

* Authors' own results

In the experiments described above, the action of highly specific L-histidine-ammonia-lyase resulted in the formation of metabolites, namely, ammonia and urocanic acid. Ammonia is the terminal product of nitrogen metabolism in humans and animals; nevertheless, it is highly toxic. Therefore, ammonia concentration should be maintained at a low level (the normal level is below 60 μM), and consequently, a technology for the removal of biotransformation products from the hydrolyzates obtained has been developed.

Removal of the by-products of histidine biotransformation (ammonia and urocanic acid) from the casein hydrolyzates resulted in the development of a technique compliant with the requirements concerning histidine-free foods or foods with a low content of histidine [8].

A two-stage process was used for hydrolyzate purification, with ammonia removal performed at the first stage. According to published data, ammonia is a colorless gas with a characteristic pungent smell, readily soluble in water, and with a density almost twice lower than that of the air. As the temperature increases, the solubility of ammonia decreases and evaporation occurs. Therefore, an attempt to delineate the optimal parameters of ammonia elimination by varying the temperature and the duration of the process at a pressure of 0.75 MPa has been undertaken. The results of the experiments are shown in Fig. 4.

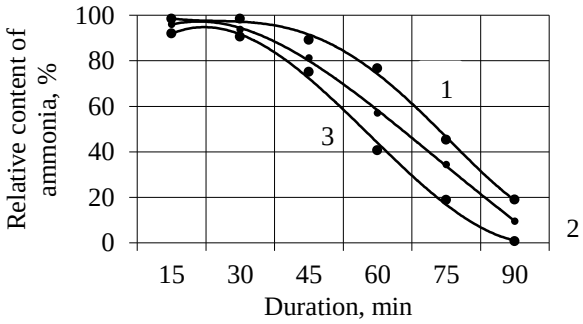


Fig. 4. Dynamics of ammonia elimination during the purification process: 1–at 60 ± 5°C; 2–at 70 ± 5°C; 3–at 80 ± 5°C.

The data obtained show that the content of ammonia is reduced by 76.5% at 60 ± 5°C and by 89.5 % at 70 ± 5°C; this does not provide for the removal of ammonia to a suffuciently high extent, and therefore the purification parameters named above cannot be used in a procedure for the production of a milk protein substitute for specialized functional foods conforming to the standards currently in force [8].However, ammonia is completely eliminated from the reaction mixture after 90 minutes of treatment at 80 ± 5°C.

Removal of urocanic acid from the hydrolyzates obtained constituted the second step of the procedure. Urocanic acid is removed from solutions by precipitation at low temperature. Therefore, subsequent experiments were aimed at selecting conditions (temperature and duration of treatment) for the most efficient removal of urocanic acid.

The results of the experiments are presented in Fig. 5.

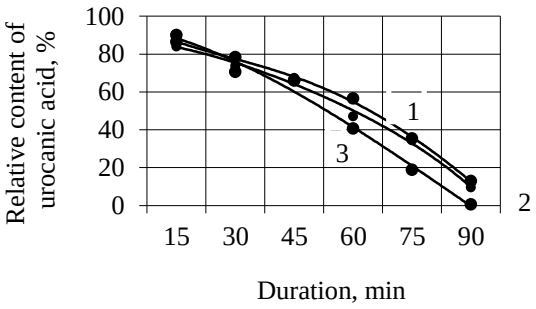


Fig. 5. Dynamics of urocanic acid removal during the purification process: 1–at a temperature of 60 ± 5°C, 2–at 70 ± 5°C, 3–at 80 ± 5°C.

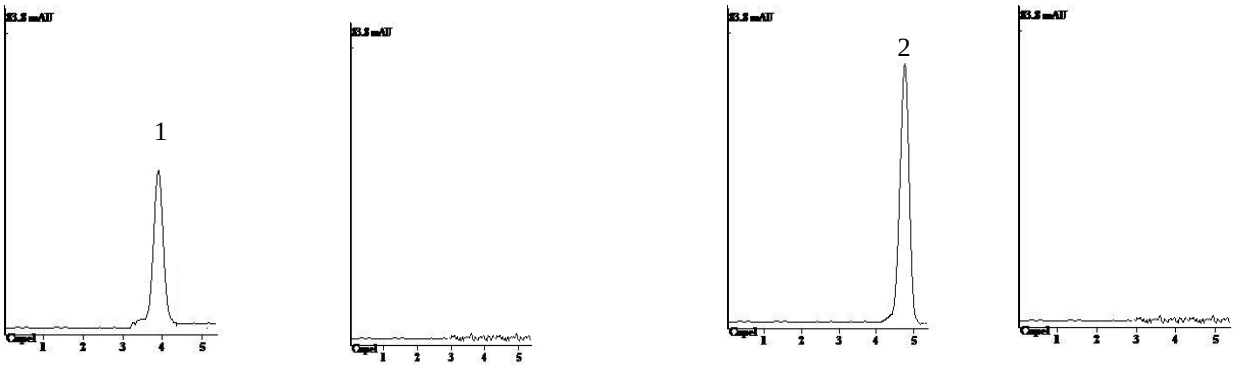


Fig. 6. Absorption spectra: 1–ammonia, 2– urocanic acid; a) before purification, b) after purification.

Therefore, one can conclude that the temperature of 80 ± 5°C and the process duration of 90 ± 1 min are the optimal conditions for the removal of urocanic acid. The absorption spectra of ammonia and urocanic acid before and after purification are shown in Fig. 6.

Table 2. The content of ammonia and urocanic acid before and after purification

Name	Content, mg/100 g	
	Before purification	After purification
Ammonia	3.27±0.05	0.001±0.0001
Urocanic acid	5.64±0.03	0.001±0.0001

The content of the biotransformation products in the hydrolyzates obtained is shown in Table 2.

The data presented in Table 2 show that the parameters selected for the treatment of the hydrolyzates obtained allow for complete removal of histidine biotransformation products from the reaction mixture and therefore the hydrolyzates obtained can be used as a milk protein equivalent in the manufacturing of specialized dietary products for histidinemia patients.

Further research will be focused on the development of a technology for the production of a milk protein equivalent for use in specialized dietary products for the nutrition of histidinemia patients.

REFERENCES

1. Prosekov, A.Yu. and Kurbanova, M.G., Analiz sostava i svoistv belkov moloka s tsel'yu ispol'zovaniya v razlichnykh otraslyakh pishchevoi promyshlennosti (Analysis of the composition and properties of milk proteins for use in various sectors of the food industry), *Tekhnika i tekhnologiya pishchevyh proizvodstv (Engineering and technology in food industry)*, 2009, no. 4, pp. 68a–71.
2. Babich, O.O., Kozlova, O.V., Razumnikova, I.S., and Prosekov, A.Yu., Biologicheski aktivnye peptidy iz belkov moloka (Biologically active peptides from milk proteins), *Molochnaya promyshlennost'* (*Dairy industry)*, 2010, no. 9, pp. 68–69.
3. Barashnev, Yu.I., Baharev, V.A., and Novikov, P.V., *Diagnosis and Treatment of Congenital Genetic Disorders in Children. A Guide to Clinical Genetics (Diagnosis and treatment of congenital genetic disorders in children. A guide to Clinical Genetics)*, Moscow: Triada-X, 2004.

4. Barashnev, Yu.I. Rozanov, A.V., Petrova, L.A., et al., Vrozhdennye poroki razvitiya golovnogo mozga, vyyavlyaemye u plodov i novorozhdennykh (Congenital malformations of the brain detected in fetuses and newborns), *Rossiiskii vestnik perinatologii i pediatrii* (*Russian Journal of Perinatology and Pediatrics)*, 2005, no. 6, pp. 9–12.
5. Berezov, T.V. and Korovin, B.F., *Bioorganicheskaya khimiya* (Bioorganic Chemistry), Moscow: Meditsina, 1990.
6. Borovik, T.E., Roslavtseva, E.A., and Gmoshinsky, I.V., Ispol'zovanie spetsializirovannykh produktov na osnove gidrolizatov belka v pitanii detei s pishchevoi allergiei. Allergologiya (The use of specialized products based on protein hydrolysates in the diet of children with food allergies. Allergology), *Pediatriya* (*Pediatrics-Moscow)*, 2001, no. 2, pp. 38–42.
7. Borovik, T.E., Makarova, S.G., Gamaleeva, A.V., and Kazakova, S.N., Spetsializirovannye smesi v profilaktike i lechenii pishchevoi allergii u detei (Specialized mixtures for the prevention and treatment of food allergies in children). *Consilium medicum. Pediatriya* (*Pediatrics-Moscow)*, 2008, no. 1, pp. 64–68.
8. Varfolomeev, S.D., *Khimicheskaya enzimologiya* (Chemical Enzymology), Moscow: Izdatel'skii tsentr "Akademiya", 2005, p. 480.
9. Rybakova, E.P., Bushueva, T.V., Ladodo, K.S., et al., Dietoterapiya nasledstvennykh narushenii aminokislotnogo obmena (Diet therapy of inherited disorders of amino acid metabolism), *Voprosy detskoi dietologii (Problems of pediatric nutritiology)*, 2005, vol. 3, no. 1, pp. 11–17.
10. Nielsen, P.M. Casein hydrolysate and method for production of such casein hydrolysate, European Patent № 0.610.411.B1, 1994.
11. Ernster, J.H., Milk protein hydrolysate and process of preparation, U.S. patent № 4600588, 1986.
12. *Gigienicheskie trebovaniya bezopasnosti i pishchevoi tsennosti pishchevykh produktov. SanPiN 2.3.2.1078-01. (Hygienic safety and nutritional value of foods. Sanitary rules and regulations 2.3.2.1078-01)*, Moscow: Ministry of Health of the Russian Federation, 2002.
13. *Spetsializirovannye produkty pitaniya dlya detei s razlichnoi patologiei. Katalog. (Specialized food products for children with different pathologies. Catalog)*, Ladodo, K.S. and Sazhinov, G.Yu., Eds., Moscow: Ministry of Agriculture of the Russian Federation, 2000.
14. Armstrong, M.D., Maternal histidinaemia, *Arch. Dis. Child.*, 1975, pp. 831–832.
15. Givot, I.L., Smith, T.A., and Abeles, R.A., Studies on the mechanism of action and the structure of the electrophilic center of histidine-ammonia-lyase, *J Biol. Chem.*, 1969, pp. 6341–6353.
16. Surhone, L.M., Tennoe, M.T. and Henssonow, S.F., Eds. *Histidine-Ammonia-Lyase*, Betascript Publishing, 2011, p. 84.
17. Levy, H.L., and Benjamin, R., Maternal histidinemia: Study of families identified by routine cord blood screening. *Pediatr Res.*, 1985, no. 19, p. 250 A.
18. Levy, H.L., Taylor, R.G., and McInnes, R.R., *Disorders of histidine metabolism*, New York: McGraw Hill, 2001, p. 1807.
19. Matsuda, I., Navada, N., and Endo, F., A family with histidinemic parents, *Pediatrics*, 1983, no. 103, p. 169.
20. Goldman, A.S., Anderson, D.W., and Sellers, W.A., Milk allergy, I: oral challenge with milk and isolated milk proteins in allergic children, *Pediatrics*, 1963, vol. 32, pp. 425–443.
21. Nowacki, P.M., Byck, S., Prevost, L., et al., Prototype for relational locus-specific mutation databases, *Nucleic Acids Res.*, 1998, pp. 220–225.
22. Schwede, T.F., Retey, J., and Schulz, G.E., Crystal structure of histidine-ammonia-lyase revealing a novel polypeptide modification as the catalytic electrophile, *Biochemistry*, 1999, p. 5355–5361.
23. Suchi, M., Sano, H., Mizuno, H., and Wada, Y. Molecular cloning and structural characterization of the human histidase gen (HAL), *Genomics*, 1995, p. 104.
24. Taylor, R.G., Levy, H.L., and. McInnes, R.R., Histidase and histidinemia. Clinical and molecular considerations, *Mol. Biol. Med.*, 1991, pp. 101–116.

IDENTIFICATION OF INDUSTRIALLY IMPORTANT LACTIC ACID BACTERIA IN FOODSTUFFS

A. Yu. Prosekov, O. O. Babich, and K. V. Bespomestnykh

Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone/fax: +7 (3842) 39-68-73, e-mail: rib@kemtipp.ru

Received May 22, 2013; accepted in revised form September 15, 2013

Abstract: Universal genus-specific primers for comparative analysis of two aligned 16S rRNA gene nucleotide sequences of lactic acid bacteria were constructed. The method to identify lactic acid bacteria and a comprehensive plan for their genus and species identification may be used to characterize isolated strains of the *Lactobacillus* genus bacteria and in quality control of foodstuffs enriched with *Lactobacillus*.

Key words: lactic acid bacteria, primers, nucleotide sequence, 16S rRNA gene, identification, PCR, phylogenetic analysis, construction

UDC 637.1.047:579.8
DOI 10.12737/2053

INTRODUCTION

Strains of the *Lactobacillus* genus, most subspecies of which transform lactose and other carbohydrates into lactic acid, are industrially important lactic acid bacteria.

This genus of microorganisms is most frequently used in production of foodstuffs of animal and plant origin by fermentation. The *Lactobacillus* genus is divided into three subspecies by the niche they occupy and their ability to ferment substances, for example, *L. delbruekcii* subsp. *delbrueckii* are usually found in vegetables treated with enzymes while *L. delbrueckii* subsp. *bulgaris* and *L. delbrueckii* subsp. *lactis*, are typically present in dairy products with addition of wide variety of carbohydrates. Specificity of industrial characteristics and vital processes makes strains of the *Lactobacillus* subspecies convenient and widely used in industry both individually and in consortium with other species to produce lactic acid dietary products, including cheese and yogurt [1, 3].

Simplicity and reliability of *L. delbrueckii* identification at various levels, that is level of strain and level of subspecies, makes them interesting not only for fundamental knowledge but for practical applications as well. Identification of microorganisms indicated on the label of a product by the manufacturer should correspond to those microorganisms that are used at a particular plant to that are applied for obtain cheese and various kinds of fermented milk products in reality.

Today, application of taxonometric identification of *L. delbrueckii* becomes urgent since there is a chance of mistake in case of utilization of phenotypic methods. These methods are based on utilization of genetic methods using strain-specific oligonucleotide probes upon blot-hybridization, DNA fingerprinting, or ribosomal DNA restriction. Today, precise and rapid identification of lactic acid bacteria is possible owing to development of methods based on polymerase chain reaction (PCR) [2, 3].

Therefore, in case rapid result is needed, methods based on PCR will be considered an alternative to microbiology tests and find wide application. Application of PCR methods allows for high-precision identification and determination of genus and species.

In the process of construction of genus- and species-specific primers, typically, 16S or 23S rRNA gene fragments, specific for each species and genus, and hypervariable internal transcribed regions (ITS), separating the above-mentioned loci, are chosen [4]. Also, transaldolase gene, small recA protein gene involved in recombination of homologous DNA, or tuf-gene coding for Tu elongation factor may serve as templates [5, 6].

In various species of *Lactobacillus*, the 16S rRNA genetic determinant has approximately the same size of about 1500 bp and is present in bacterial genome in several copies. Spacer regions of various size serve as separators between the copies of bacterial genes. Presence of two flanked spacer regions different in size between 16 and 23S rRNA genes is typical of the *Lactobacillus* genus. The 16S rRNA gene, containing both variable and conservative regions in the nucleotide sequence, is the most appropriate for determination of genus and species affiliation of a bacterium.

Therefore, the aim of the work was to construct genus- and species-specific primers to detect and identify industrially important strains of the *Lactobacillus* genus.

MATERIALS AND METHODS

In the work we used ten bacterial strains of the *Lactobacillus* genus registered in the All-Russian Collection of Industrial Microorganisms (VKPM; FGUP GosNII-genetika), five of which, according to the passport data, belong to the *L. acidophilus* species, and the other five, to *L. delbruesckii* ssp. *bulgaricus* (Table 1), and 12 nucleotide sequences of 16S rRNA genes of lactic acid bacteria deposited in the NCBI data base (Table 2).

Table 1. Microorganisms of the All-Russian Collection of Industrial Microorganisms (VKPM; FGUP GosNII-genetika)

No.	VKPM ID	Strain title	Source
1	V-6551	L.a. T-3	Feces of a newborn
2	V-8153	L.a. AE-5	Obtained from the L.a. AT-44 strain
3	V-194	L.a.1k	Isolated from chicken
4	V-8634	L.a. 3	Self-fermented curdled milk
5	V-5863	L.a. 57S	Self-fermented milk
6	V-6516	L.d. b. 21	Isolated from milk
7	V-3964	L.d. b.	Raw milk
8	V-3141	L.d. b. L20/2	Matzoon
9	V-6543	L.d. b. B-259	Self-fermented milk product
10	V-6515	L.d. b. 19	Isolated from milk

Table 2. Record numbers of 16S rRNA gene sequences of the *Lactobacillus* genus representatives deposited in NCBI database

Record number of 16S rRNA gene sequence in NCBI	Strain title
CP000033.3	*Lactobacillus acidophilus* NCFM
FJ556999.1	*Lactobacillus acidophilus* strain CECT 4529
FJ749655.1	*Lactobacillus acidophilus* strain IMAU30067
EU878007.1	*Lactobacillus acidophilus* strain NX2-6
AY763429.1	*Lactobacillus acidophilus* strain LH4
FJ861093.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain KLDS 1.0625
EU483107.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain LC
CP000412.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365
EU642554.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain IMAU40169
FJ915706.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain IMAU40169
EU547306.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain BCS113
AF429503	*Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842

Isolation of DNA. DNA from dry bacteria culture was isolated using the Kit for Isolation of Genomic DNA from Bacteria (Sintol, Moscow). To extract bacterial DNA, cells were sedimented from 1 mL liquid culture by centrifugation at 10000 rpm for 3 min and the supernatant was removed. Then, cells were resuspended in 300 μL buffer (10 mM Tris HCl, pH 8.0; 50 mM glucose, 10 mM EDTA) and 3 μL lysozyme (10 mg/mL) was added to the suspension. Cell wall was lysed at 37°C for 60 min under occasional stirring of the tube content by overturning it, then the suspension was centrifuged at 13000 rpm for 1 min. The sediment was resuspended in 300 μL of lysing buffer (20 mM Tris-HCl, pH 8.0; 75 mM NaCl; 1% SDS; 10 mM EDTA) and 3 μL RNase A (10 mg/mL) was added to the mixture. Incubation was performed at 37°C for 30 min, then the mixture was cooled on ice for 1 min. Then, 100 μL solution for protein precipitation (7.5 M ammonium acetate) was added to the mixture and mixed on a vortex for 20 s, then it was centrifuged at 13000 rpm for 5 min. The supernatant was transferred into clean 1.5 mL tubes and 300 μL isopropanol were added, the mixture was mixed by overturning for 1 min and placed at –20°C for 30 min. The mixture was centrifuged at 13000 rpm for 5 min, the supernatant was accurately decanted, and the tubed were placed overturned on a clean filter. Then, 400 μL of 70% ethanol was added and mixed several times by overturning to wash the DNA sediment. The procedure was repeated several times. Finally, the sediment was dried at 37°C 15 min till ethanol drops disappeared completely. The dried sediment was dissolved in 30 μL TE buffer.

Phylogenetic analysis. To construct species-specific and genus-specific primers, 12 nucleotide sequences of 16S rRNA gene from lactic acid bacteria deposited in the NCBI database were used: *Lactobacillus acidophilus* CP000033.3, FJ556999.1, FJ749655.1, EU878007.1, and AY763429.1; *Lactobacillus delbruesckii* ssp. *bulgaricus* FJ861093.1, EU483107.1, CP000412.1, EU642554.1, FJ915706.1, and EU547306.1 (for species-specific primers); *Lactobacillus delbruesckii* ssp. *bulgaricus* AF429503 and *Lactobacillus acidophilus* AY763429 (for genus-specific primers).

Analysis of DNA sequence coding for 16S rRNA synthesis is the basis for phylogenetic analysis of bacteria. To determined differences in sequences, comparative analysis and construction of phylogenetic tree were performed in ClustalW software.

Oligonucleotide synthesis was performed on an ASM1000 (Biosset, Novosibirsk) DNA/RNA synthesizer. Purification was performed in a polyacrylamide gel.

Amplification of 16S rRNA gene was performed on a Tertsyk equipment (Moscow) using a thermostable Taq polymerase (SibEnzim, Nosvosibirsk) according to the manufacturer's recommendations. The synthesized primers were used for amplification.

Temperature–time profile of PCR was the following: 95°C for 200 s, one cycle; 62°C for 50 s and 95°C for 20 s, 25 cycles; and 72°C for 120 s, one cycle. Analysis of PCR products was performed using electrophoresis in 1.5% gel, containing ethidium bromide. The results were documented using a Vitran (Biokom) videosystem. DNA fragment length marker was used.

RESULTS AND DISCUSSION

To construct universal genus-specific primers, comparative analysis of two aligned nucleotide sequences of 16S rRNA genes from lactic acid bacteria was performed. Results of the analysis allowed for construction of universal primers for detection of the *Lactobacillus* genus bacteria on the basis of 16S rRNA gene,

16S for: 5'- AGA GTT TGA TCC TGG CTC AGG A
and
16S rev: 5'- ACG CTT GCC ACC TAC GTA TTA C,

for amplification of 566 bp-long 16S rRNA gene sequence.

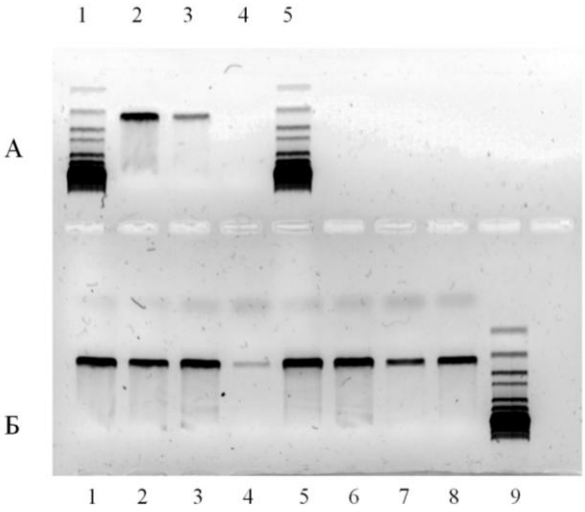


Fig. 1. Electrophoregram of lactic acid bacteria 16S rRNA gene fragment amplification products using synthetic primers: (A) *1*, Medigen 400–2500 bp weight marker, *2*, no. 9, *3*, no. 10, *4*, negative control (H_2O), *5*, marker; (B) *1*, no.1, *2*, no. 2, *3*, no. 3, *4*, no. 4, *5*, no. 5, *6*, no. 6, *7*, no.7, *8*, no. 8, and *9*, marker.

Theoretical specificity of genus-specific primers was confirmed experimentally in ten strains, five of them belonging to *L. acidophilus* species, and another five, to *L. delbrueckii* ssp. *bulgaricus*. Synthesized primers were used in amplification of the 16S rRNA gene fragment in strains under study (Fig. 1).

The oligonucleotides may form basis for a test system allowing for *Lactobacillus* bacteria detection.

Synthesis of primers for amplification of 16S fragments of lactobacilli allowed for elaboration of a fragment, the size of which is sufficient for genomic characteristics elucidation.

To construct species-specific primers, comparative analysis of 11 complete nucleotide sequences of 16S rRNA fragments of lactic acid bacteria was performed using a ClustalW software.

For this purpose, we searched the database of complete nucleotide sequences corresponding to 16S rRNA genes of reference bacteria strains of the *Lactobacillus* genus and chose *Lactobacillus acidophilus* sequences deposited in NCBI database under record numbers CP000033.3, FJ556999.1, FJ749655.1, EU878007.1, and AY763429.1, and *Lactobacillus delbruesckii* ssp. *bulgaricus* sequences FJ861093.1, EU483107.1, CP000412.1, EU642554.1, FJ915706.1, and EU547306.1.

The chosen nucleotide sequences were used for comparative phylogenetic analysis, the results of which are presented in Fig. 2.

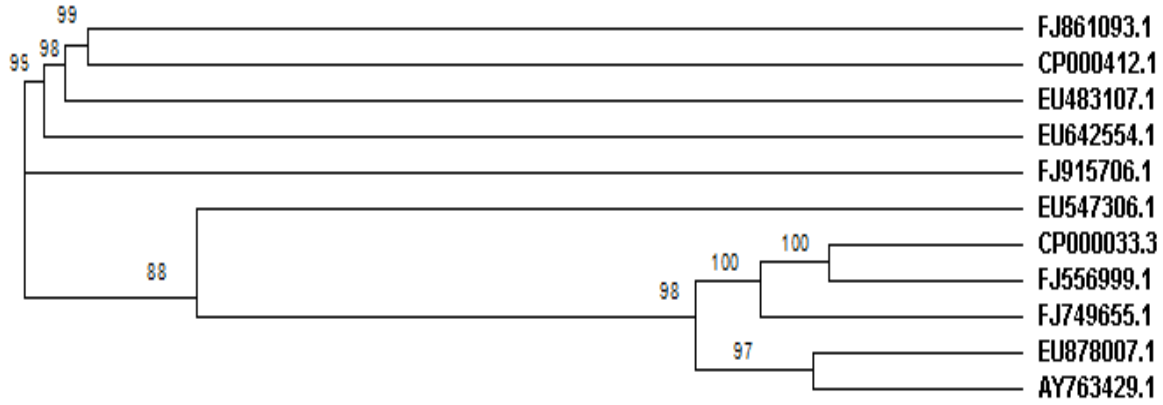


Fig. 2. Phylogenetic tree built based on analysis of 16S rRNA fragment sequences reflecting relationships between lactic acid bacteria *L. bulgaricus* and *L. acidophilus* under study.

The eleven 16S rRNA gene sequences of various lactobacilli that we used were grouped into four clusters: the first cluster contained type strains of the *L. acidophilus* species (CP000033.3, FJ556999.1, FJ749655.1, EU878007.1, and AY763429.1); the second and the third, strains of the *L. bulgaricus* species (EU547306.1 and FJ915706.1, respectively); and the fourth one, strains of the *Lactobacillus delbruesckii* ssp. *bulgaricus* species (FJ861093.1, EU483107.1, CP000412.1, and EU642554.1). The phylogenetic tree (Fig. 1) was built using the ClustalW software. As follows from the data presented in Figure 1, in the first and the fourth clusters two closely related species, *L. acidophilus* and *Lactobacillus delbruesckii* ssp. *bulgaricus*, are joined. According to the data obtained upon the analysis of complete nucleotide sequences of 16S rRNA gene fragments, homology of gene structure between different representatives of the *Lactobacillus* genus with respect to 16S rRNA gene of *Lactobacillus delbruesckii* ssp. *bulgaricus* is 87–93% for the *L. acidophilus* species and 92–99%, for the *L. bulgaricus* strains.

Comparative analysis demonstrated that the most important differences in sequences coding for 16S rRNA are observed in the first third of the sequence. Earlier, foreign authors have systematized species of the *Lactobacillus* genus according to their 16S rRNA genes and other marker sequences.

We optimized the primer sequences proposed previously in order to increase the temperature of annealing and improve specificity of the obtained PCR product. As a result, the following species-specific primers were synthesized,

16SbulF: 5'- CAA CAG AAT CGC ATG ATT CAA GTT TG (26) and

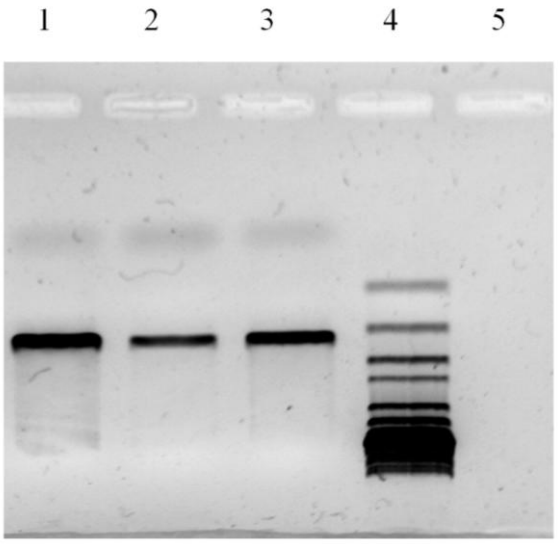


Fig. 3. Electrophoregram of amplification products of genome fragments from different representatives of the *Lactobacillus* genus contained in various substrates: *1*, yogurt; *2*, Bifilaif; *3*, positive control; *4*, molecular weight marker; *5*, negative control.

16SbulR: 5'- ACC GGA AAG TCC CCA ACA CCT A (22),

for amplification of 675 bp-long 16S rRNA sequences.

The newly developed primers possess low homology to the corresponding DNA regions of other lactic acid bacteria, which provides for their specificity. Theoretical specificity of the primers was confirmed experimentally in probiotic products (Fig. 3).

In frames of the research topic, the step of species identification is performed using isolated fragments, which allows for rapid detection of bacteria of the *Lactobacillus* genus in a sample and identification of the *L. acidophilus* and *L. delbruesckii* ssp. *bulgaricus* species. The latter ones are contained in probiotics and sanative foodstuffs and are industrially important. The developed method of detection of lactic acid bacteria and the comprehensive plan for their identification may be used for characteristics of isolated strains of the *Lactobacillus* genus and is of practical interest for applications in quality control of foodstuffs enriched with lactobacilli.

ACKNOWLEDGMENTS

The work was supported by the Federal Target Program "Research and Academic Staff of Innovative Russia" in the years 2009–2013.

REFERENCES

1. Tochilina, A.G., Indikatsyya i identifikatsyya bakterii roda lactobacillus s ispol'zovaniem polimeraznoi tsepnoi reaktsii (Detection and identification of bacteria of the Lactobacillus genus using polymerase chain reaction), *Mikrobiologiya, epidemiologiya i immunobiologiya (Microbiology, Epidemiology, and Immunobiology)*, 2008, no. 3, pp. 69–73.
2. Botina, S.G., Identifikatsyya promyshlennykh shtammov molochnokislykh bakterii metodami molekulyarno-geneticheskogo tipirovaniya (Identification of industrial strains of lactic acid bacteria by methods of molecular genetics typing), *Genetika (Genetics)*, 2006, vol. 42, no. 12, pp. 1621–1635.
3. Torriani, S., Zapparoli, F., and Dellaglio, F., Use of PCR-Based methods for rapid differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*, *Appl. Environ. Microbiol.*, 1999, vol. 65, no. 10, pp. 4351–4356.
4. Heilig, H.G., Zoetendal, E.G., Marteau, P, et al., Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA, *Appl. Environ. Microbiol.*, 2002, vol. 68, no. 1, pp. 114–123.
5. Torriani, S., Felis, G.E., Dellaglio, F., Differentiation of *Lactobacillus plantarum, L. pentosus,* and *L. paraplantarum* by *recA g*ene sequence analysis and multiplex PCR assay with *recA g*ene-derived primers, *Appl. Environ. Microbiol.*, 2001, vol. 67, no. 8, pp. 3450–3454.
6. Ventura, M., Canchaya, C., Meylan, V., et al., Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification, *Appl. Environ. Microbiol.*, 2003, vol. 69, no. 11, pp. 6908–6922.

INVESTIGATION OF THE BIOTECHNOLOGICAL ACTIVITY OF DIRECT-SET STARTER CULTURES IN STRUCTURED DAIRY PRODUCTS

A. N. Arkhipov, A. V. Pozdnyakova, and K. A. Shevyakova

Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone/fax: +7 (3842) 39-68-96, e-mail: anjap12@rambler.ru

Received April 10, 2013; accepted in revised form November 26, 2013

Abstract: Galactosidase and proteinase activity of lactic acid bacteria of the DELVO-YOG range has been investigated. Lactic acid bacteria of the DELVO-YOG range have been shown to exhibit maximal galactosidase activity in the presence of the CMC stabilizer Akucell 2785; the activity was minimal when sodium alginate NO4-600 was used as a stabilizer. The proteolytic activity demonstrated by the lactic acid bacteria of the DELVO-YOG range was maximal when sodium pyrophosphate SAPP 28 was used as a stabilizer. Minimal proteolytic activity of lactic acid bacteria of the DELVO-YOG range was registered when CMC 6000-9000 was used as a stabilizer. An increase of galactosidase and proteinase activity concomitant to an increase of the stabilizer content from 0.5 to 1.0 mass. % was demonstrated for all the denominations of lactic acid bacteria of the DELVO-YOG range.

Key words: milk, lactic acid bacteria, activity, glycolysis, proteolysis, structure stabilizer

UDC 634.146.33
DOI 10.12737/2054

INTRODUCTION

Functional food comprises special-purpose products of natural or artificial origin with predefined properties; these products are intended for systematic everyday use and their primary role is to compensate for the deficiency of the regulatory components of food in the organism. Functional foods contribute to health preservation, disease prevention, and enhancement of the body's ability to withstand adverse environmental effects and to endure physical and psycho-emotional stress. The significance of nutrition is emphasized by the Decree № 917 of 10.08.98 "On the Concept of the State Policy Regarding Healthy Nutrition of the Population of the Russian Federation" of the Government of Russian Federation.

Fermented dairy products are manufactured using starter cultures of a defined composition. Milk is an ideal nutrient medium for starter cultures, which are used to direct and control biochemical transformations. Biochemical transformation of milk components includes controlled glycolysis of lactose yielding lactic acid and regulated proteolysis of casein, and therefore both galactosidase activity and proteolytic activity are important characteristics of the starter cultures [1]. The most useful species of starter cultures must display maximal activity during fermentation.

The structure of stabilized dairy products is formed as a result of casein coagulation as the isoelectric point is reached. Structure stabilizers allow for structure formation at pH values deviating from the isoelectric point value. Moreover, the use of stabilizers allows for control over the structural, mechanical, physico-chemical, and organoleptic parameters of the final product, as well as its general quality. Stabilizers are widely used to regulate the structure of fermented milk beverages and achieve the required texture which does not change during storage and remains stable throughout the technological process. Stabilizers should prevent delamination of the product and separation of whey; in case of yoghurts containing fillers and additives stabilizers should provide for uniform distribution of these components in the product during packing and subsequent storage.

The main objectives of the manufacturing of structured fermented dairy products are the optimization of stabilizer content, the selection of starter cultures possessing high acid-forming capacity, and the selection of cultivation conditions providing for rapid propagation of the microorganisms and intensive acid production by them. Cultivation of starter cultures that have significant proteolytic activity accelerates the propagation of bifidobacteria in the structured product and improves the organoleptic properties of the product. A wide range of starter cultures adapted to specific features of the technology used and the biochemical processes of the formation of texture and taste properties of dairy products is available. The qualitative composition of starter cultures is being constantly improved [2].

Food stabilizers are currently considered to be among the most important components of the majority of structured fermented dairy products, since they allow for the manufacturing of a product of required texture [3]. In addition to improving the product quality and increasing its shelf life, the use of stabilizers allows for a decrease of product costs, this improving the economic performance of the enterprise. The experiments performed in the present study revealed the decisive role of the environmental conditions (mass concentration and type of the stabilizer) in regulating the galactosidase and proteinase activity of starter cultures. Investigation of the dependence of galactosidase and proteolytic activity of DELVO-YOG starter cultures on the type and content of the stabilizer can be of practical importance for the production of stabilized dairy products.

OBJECTS AND METHODS OF THE STUDY

The study was carried out with the following objects:

- Mesophilic aramate-forming DELVO-YOG starter cultures CY-346/347, FVV-21, CY DSL, and FVV-31;
- Structure stabilizers: CMC Akucell 3265, CMC 4500-6000, konjac gum, CMC 6000-9000, pectin ARA 105, carob gum, sodium alginate NO4-600, sodium pyrophosphate SAPP 28, CMC Akucell 2785, sodium pyrophosphate SAPP 40, and xanthan gum;
-Potable water conforming to GOST (State Standard) 2874;
- Structured fermented dairy products with different composition and properties produced either in the laboratory or industrially;
- Raw cow milk (grade II or higher according to GOST (State Standard) 13264);
- Whey conforming to OST (Industrial Standard) 4992;
- Whey powder conforming to TU (Technical Specifications) 49800;
- Granulated sugar conforming to GOST (State Standard) 21;
- Auxiliary materials conforming to the regulatory documents currently in force.

Conventional, standard and original methods were used in the study.

Sampling of milk and milk products and preparation of samples for analysis were performed in accordance with GOST (State Standard) 26809-86.

Sampling for microbiological testing was performed in accordance with GOST (State Standard) 9225-84.

Titration-based acidity assay was performed in accordance with GOST (State Standard) 3524-92.

Active acidity was measured on a potentiometric analyzer according to GOST (State Standard) 26781-85.

Evaluation of taste and smell was performed in accordance with GOST (State Standard) 28283-92.

Total protein content was assayed in accordance with GOST (State Standard) 23327-78.

Activity of the enzyme system referred to the difference between the non-protein nitrogen or lactose concentration measured in non-fermented whey and the respective concentration measured in a system consisting of hydrolyzed whey and native whey pretreated with fermenting microorganisms. The average values of galactosidase activity and proteolytic activity of the lactic acid bacteria of DELVO-YOG series measured in samples with different stabilizer content (mass %) were analyzed in the present work. The stabilizer content was increased from 0.5 to 2.5 mass % in steps of 0.5 mass %.

Galactosidase activity of lactic acid bacteria was determined as follows: a 5% solution of lactose in a buffer solution (pH 4.2 or 7.0) and a 1% solution of the enzyme were prepared, and the enzyme activity was determined using the freezing point depression method. For this, 1 cm^3 of the enzyme solution and 4 cm^3 of the substrate solution were mixed, and a 1 cm^3 aliquot (control) was drawn from the mixture. The remaining solution was incubated at 30° C for 30 minutes, and after this a 1 cm^3 test aliquot was drawn and its freezing temperature was measured and used to calculate galactosidase activity.

A method based on the hydrolysis of sodium caseinate by the enzyme preparation under investigation with subsequent quantitation of the peptides formed was used for the determination of proteolytic activity. A unit of proteolytic activity was defined as the ability of the enzyme to convert sodium caseinate into compounds not precipitated by trichloroacetic acid in an amount equivalent to 1 micromole of tyrosine (GOST (State Standard) 20264.2-88) in one minute at 30°C. The activity of proteolytic enzymes in the medium and in the cells was evaluated by measuring the difference in the content of ninhydrin-positive substances in the reaction mixture. The method involves monitoring of the changes in the content of ninhydrin-positive substances in the reaction mixture with subsequent quantitative assessment; the accumulation of the extracellular enzyme of the microorganism under investigation is assayed according to the procedure developed by Chebotarev.

RESULTS AND DISCUSSION

Stabilizer content of 1.5 mass % was chosen in order to maintain the structure of the fermented dairy product; the choice was based on the analysis of the average values of galactosidase activity of lactic acid bacteria of the DELVO-YOG series.

Table 1. Galactosidase activity of the DELVO-YOG starter culture at the stabilizer content of 1.5 mass %, Δg lactose/100 mg

Stabilizer type	Starter culture name			
	CY-346/347	FVV-21	CY DSL	FVV-31
CMC Akucell 3265	3.29±0.05	3.48±0.05	3.02±0.05	3.29±0.05
CMC 4500-6000	2.81±0.05	2.94±0.05	2.98±0.05	3.14±0.05
Conjac gum	2.94±0.05	2.90±0.05	2.97±0.05	3.11±0.05
CMC 6000-9000	3.06±0.05	3.33±0.05	3.29±0.05	2.96±0.05
Pectin ARA 105	3.30±0.05	3.30±0.05	3.17±0.05	3.23±0.05
Locust bean gum	2.96±0.05	3.28±0.05	2.74±0.05	2.88±0.05
Sodium alginate NO4-600	2.77±0.05	2.16±0.05	2.58±0.05	2.79±0.05
Sodium pyrophosphate SAPP 28	2.84±0.05	2.94±0.05	2.81±0.05	2.91±0.05
CMC Akucell 2785	3.47±0.05	3.54±0.05	3.32±0.05	3.42±0.05
Sodium pyrophosphate SAPP 40	3.05±0.05	3.11±0.05	2.63±0.05	2.93±0.05
Xanthan gum	3.13±0.05	2.99±0.05	2.83±0.05	3.12±0.05

The optimal stabilizer type was chosen according to the values of galactosidase activity (Δg lactose/100 mg) of the DELVO-YOG starter culture (subtypes CY-346/347, FVV-21, CY DSL, and FVV-31) measured at a stabilizer content of 1.5 mass %. The results of the experiment are presented in Table 1.

An increase of galactosidase activity by 0.03–0.17 was registered for all types of DELVO-YOG starter culture. The galactosidase activity was maximal when CMC Akucell 2785 was used as a stabilizer; the activity amounted to 3.47 ± 0.05 Δg lactose/100 mg for

CY-346/347, 3.54 ± 0.05—for FVV-21, 3.32 ± 0.05—for CY DSL, and 3.42 ± 0.05—for FVV-31. The activity was minimal when sodium alginate NO4-600 was used as the stabilizer.

The dependence of galactosidase activity of DELVO-YOG lactic acid bacteria on the content of CMC 6000-9000 was analyzed in order to determine the optimal content of the stabilizer. The results of the experiment are presented in Fig. 1.

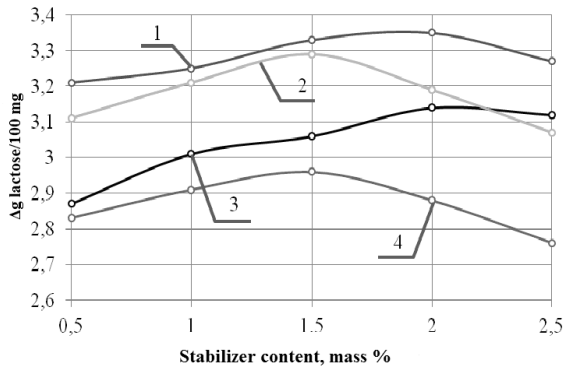


Fig. 1. Dependence of galactosidase activity of the DELVO-YOG starter culture on the content of CMC 6000-9000, mass %: 1–FVV-21; 2–CY DSL; 3–CY-346/347; 4–FVV-31.

Maximal increase in galactosidase activity of the DELVO-YOG starter culture was observed as the content of the stabilizer was increased from 1.5 to 2.0%.

The dependence of galactosidase activity of DELVO-YOG CY-346/347 starter culture on the stabilizer type at the stabilizer concentration of 1.5% was analyzed to identify the optimal type of stabilizer.

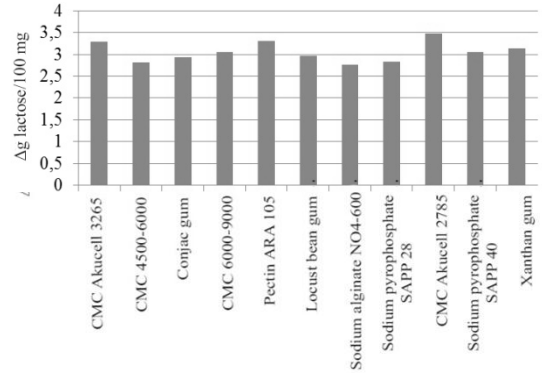


Fig. 2. Galactosidase activity of DELVO-YOG CY-346/347 lactic acid bacteria at a stabilizer content of 1.5 mass %.

DELVO-YOG CY-346/347 starter culture exhibited maximal galactosidase activity when CMC Akucell 2785 was used as a stabilizer.

Stabilizer content of 1.5 mass % was chosen according to the results of the analysis of the average proteolytic activity values of lactic acid bacteria. The proteolytic activity of DELVO-YOG starter culture at a stabilizer content of 1.5 mass % was analyzed in order to choose the optimal stabilizer. The results of the experiments are presented in Table 2.

Table 2. Proteolytic activity of lactic acid bacteria of the DELVO-YOG series at a stabilizer content of 1.5 mass %, 10^{-2} Δmg non-protein nitrogen/100 mg

Stabilizer type	Name			
	CY-346/347	FVV-21	CY DSL	FVV-31
CMC Akucell 3265	2.57±0.05	2.52±0.05	2.70±0.05	2.54±0.05
CMC 4500-6000	2.93±0.05	2.88±0.05	2.98±0.05	3.06±0.05
Konjac gum	2.70±0.05	2.63±0.05	2.76±0.05	2.71±0.05
CMC 6000-9000	2.51±0.05	2.43±0.05	2.31±0.05	2.52±0.05
Pectin ARA 105	3.16±0.05	3.02±0.05	3.03±0.05	3.24±0.05
Locust bean gum	2.78±0.05	2.63±0.05	2.82±0.05	3.00±0.05
Sodium alginate NO4-600	2.93±0.05	2.73±0.05	2.70±0.05	2.86±0.05
Sodium pyrophosphate SAPP 28	3.29±0.05	3.06±0.05	3.11±0.05	3.27±0.05
CMC Akucell 2785	3.00±0.05	2.89±0.05	2.94±0.05	2.98±0.05
Sodium pyrophosphate SAPP 40	2.59±0.05	2.42±0.05	2.65±0.05	2.79±0.05
Xanthan gum	2.79±0.05	2.80±0.05	2.76±0.05	2.70±0.05

The values of the proteolytic activity of lactic acid bacteria increased by 0.01–0.05; maximum proteolytic activity of starter cultures, amounting to 3.29 ± 0.05 for CY-346/347, 3.06 ± 0.05 for FVV-21, 3.11 ± 0.05 for CY DSL, and 3.27 ± 0.05 for FVV-31, was registered when sodium pyrophosphate SAPP 28 was used as a stabilizer. Proteolytic activity was the lowest when CMC 6000-9000 was used as a stabilizer: in this case it amounted to 2.51 ± 0.05 for CY-346/347, 2.43 ± 0.05 for FVV-21, 2.31 ± 0.05 for CY DSL, and 2.52 ± 0.05 for FVV-31.

The dependence of the proteolytic activity of the DELVO-YOG starter culture on the content of CMC Akucell 3265 was analyzed in order to determine the optimal content of the stabilizer. Results of the experiment are presented in Fig. 3: 1–CY-346/347; 2–FVV-31, 3–FVV-21, 4–CY DSL.

An increase of the proteolytic activity of DELVO-YOG starter cultures (with the exception of FVV-21) was observed as the stabilizer content was increased from 1.5 to 2.0 mass %.

The dependence of the proteolytic activity of DELVO-YOG CY-346/347 lactic acid bacteria on stabilizer type at the stabilizer concentration of 1.5 mass % was analyzed in order to identify the optimal type of stabilizer.

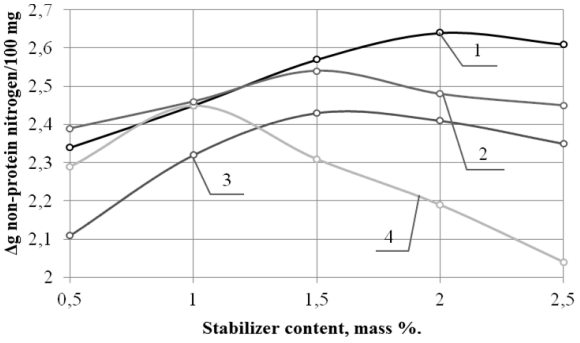


Fig. 3. The dependence of the proteolytic activity of DELVO-YOG lactic acid bacteria on the content of CMC Akucell 3265, mass %: 1–CY-346/347; 2–FVV-31, 3–FVV-21, 4–CY DSL.

Notably, both galactosidase activity and proteolytic activity of all types of DELVO-YOG starter culture increased as the stabilizer content was increased from 0.5% to 1.0 mass %.

As a rule, use of the stabilizer at a content of 1.5 mass % provided for the highest values of galactosidase activity and proteolytic activity of DELVO-YOG starter cultures

Maximal values of proteolytic activity of DELVO-YOG starter cultures were registered when sodium pyrophosphate SAPP 28 was used as stabilizer; these values amounted to 3.29 ± 0.05 for CY-346/347, 3.06 ± 0.05 for FVV-21, 3.11 ± 0.05 for CY DSL, and 3.27 ± 0.05 for FVV-31. The minimal values of the proteolytic activity of DELVO-YOG starter cultures were registered when CMC 6000-9000 was used as a stabilizer and amounted to 2.51 ± 0.05 for CY-346/347, 2.43 ± 0.05 for FVV-21, 2.31 ± 0.05 for CY DSL, and 2.52 ± 0.05 for FVV-31, as shown in Fig. 4.

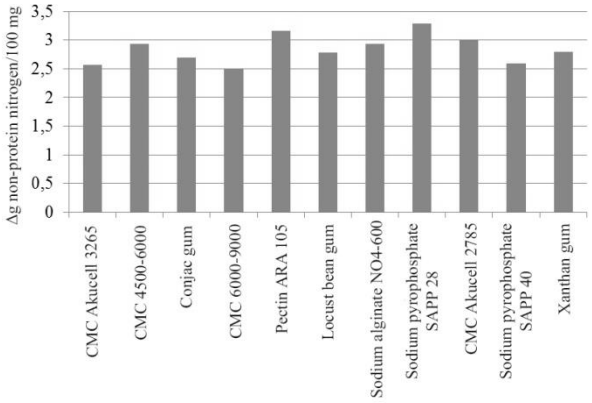


Fig. 4. Proteolytic activity of the DELVO-YOG CY-346/347 starter culture at a stabilizer content of 1.5 mass %.

The increase of galactosidase activity and proteolytic activity of lactic acid bacteria upon the increase of the stabilizer content above 1.5 mass % was insignificant (not more than 0.09 units), and in some cases a slight decrease of galactosidase activity and proteolytic activity of lactic acid bacteria was observed.

Maximal galactosidase activity of DELVO-YOG starter cultures was detected when CMC Akucell 2785 was used as a stabilizer; the activity values amounted to 3.47 ± 0.05 for CY-346/347, 3.54 ± 0.05 for FVV-21, 3.32 ± 0.05 for CY DSL, and 3.42 ± 0.05 for FVV-31, and minimal activity was registered when sodium alginate NO4-600 was used as the stabilizer.

REFERENCES

1. Prosekov, A. Yu., *Sovremennye aspekty proizvodstva produktov pitaniya* (Modern Aspects of Food Production), Kemerovo: Kuzbassvuzizdat, ASTSh, Universitety Rossii, 2005.
2. Prosekov, A. Yu. and Korotkaya, E. V., *Kriokonservirovanie bakterial'nykh preparatov molochnoi promyshlennosti* (Cryopreservation of Bacterial Preparations for Dairy Industry), Kemerovo: Kem TIPP, 2010.
3. Gorbatova, K. K., *Fiziko-khimicheskie i biologicheskie osnovy proizvodstva molochnyh produktov* (Physico-Chemical and Biochemical Basis of the Manufacturing of Dairy Products), Moscow: GIORD, 2003.

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

O. O. Babich* and E. V. Ulrikh**

*Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone/fax: +7 (3842) 68-06-83, e-mail: olich.43@mail.ru

**Kuzbass State Technical University named after T.F. Gorbatchev,
28 Spring, Kemerovo, 650000 Russia,
phone/fax: +7 (3842) 39-68-74, e-mail: elen.ulrich@mail.ru

Received February 13, 2013; accepted in revised form February 27, 2013

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

UDC 577.112.387.2:577.152.4
DOI 10.12737/2056

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).

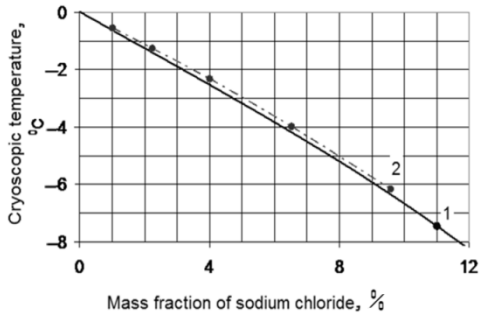


Fig. 1. Cryoscopic temperature of solution in function of sodium chloride concentration: 1, sodium chloride aqueous solution; 2, enzyme preparation.

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).

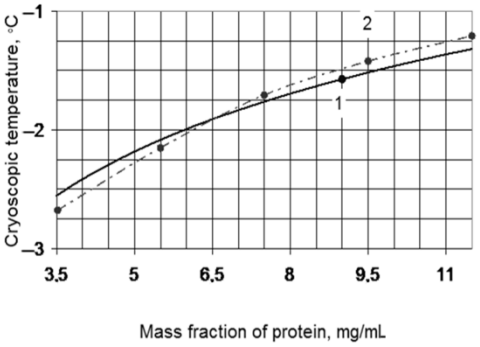


Fig. 2. Cryoscopic temperature of L-phenylalanine ammonia-lyase enzyme preparation in function of protein concentration.

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.

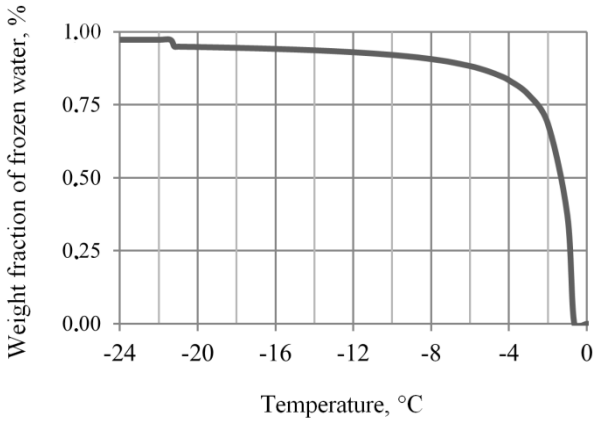


Fig. 3. Weight fraction of frozen water in L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

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.

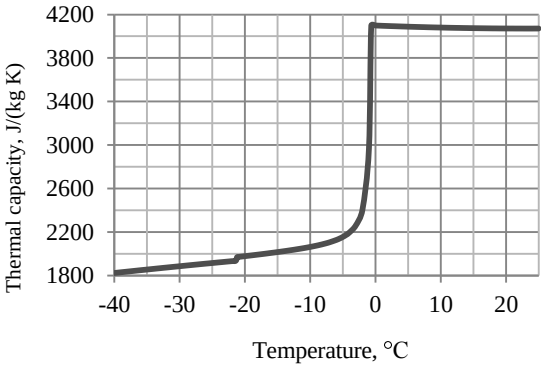


Fig. 4. Specific thermal capacity of L-phenylalanine-ammonia lyase enzyme preparation in function of temperature.

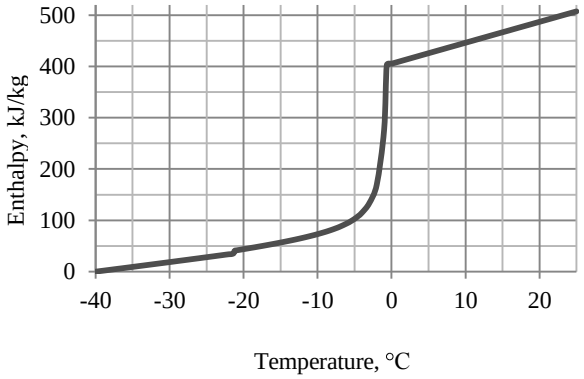


Fig. 5. Specific enthalpy of L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

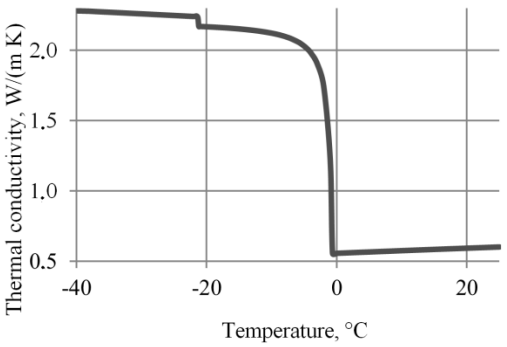


Fig. 6. Coefficient of thermal conductivity of L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

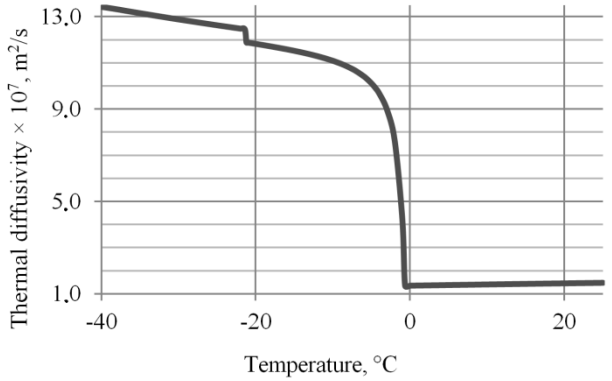


Fig. 7. Coefficient of thermal diffusivity of L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

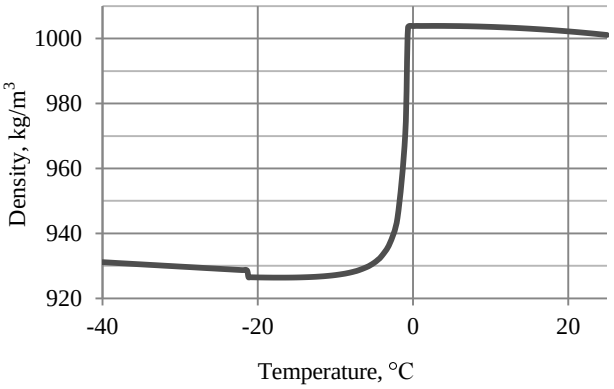


Fig. 8. Density of the L-phenylalanine ammonia-lyase enzyme preparation in function of temperature.

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.

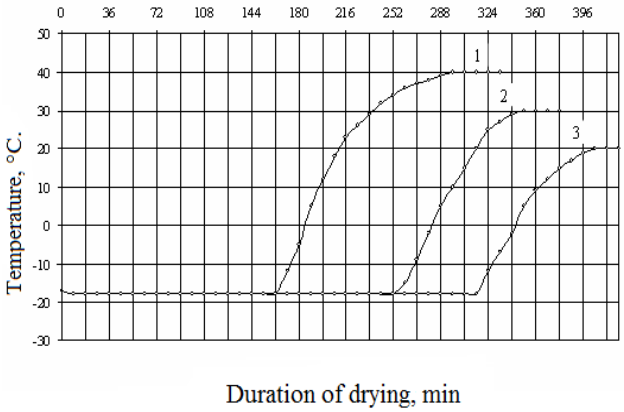


Fig. 9. Typical drying curves of the L-phenylalanine-ammonia lyase preparation under the studied levels of stabilization of dry level: 1, 40°C; 2, 30°C; and 3, 20°C.

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.

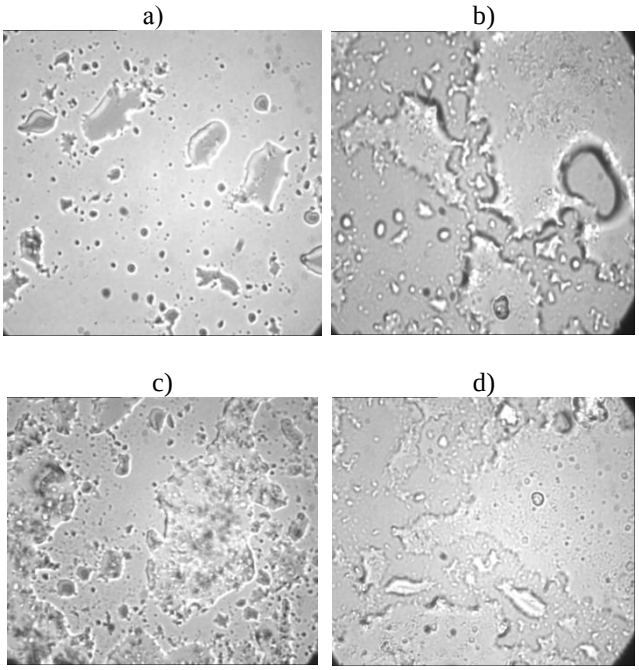


Fig. 10. Microstructure of dried enzyme preparation (magnification of 500×) at different temperatures of stabilization on surface of the dry layer: (a) 15°C; (b) 20°C; (c) 30°C; and (d) 40°C.

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%.

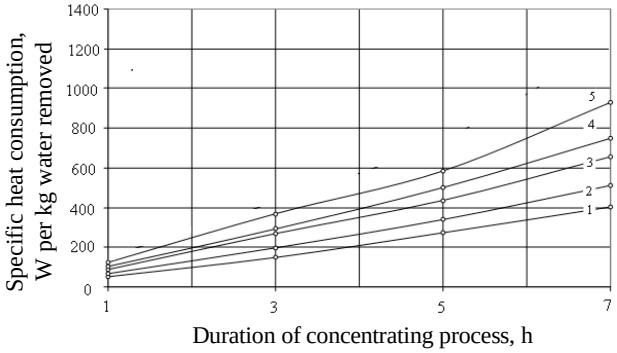


Fig. 11. Specific heat consumption during sublimation process of L-phenylalanine ammonia-lyase enzyme preparation in function of stabilization temperature of the dry layer: 1, 5°C; 2, 15°C; 3, 20°C; 4, 30°C; and 5, 40°C.

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).

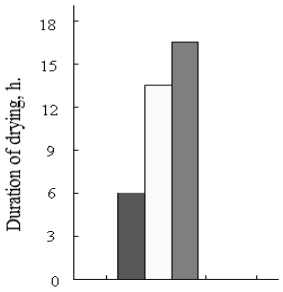


Fig. 12. Duration of sublimation drying of L-phenylalanine ammonia-lyase enzyme preparation in function of drying temperature:
- drying temperature 20ºC;
- drying temperature 30ºC;
- drying temperature 40ºC.

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.

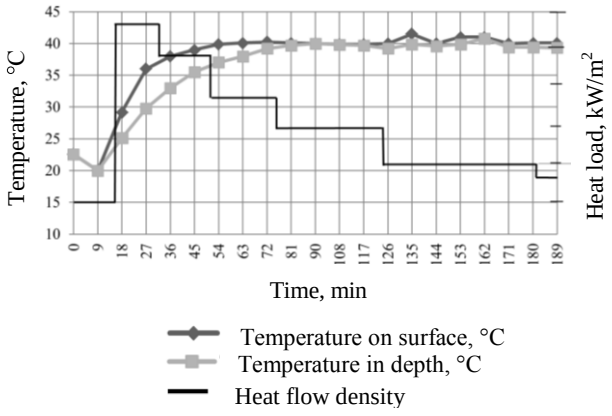


Fig. 13. Sublimation drying curves of the L-phenylalanine ammonia-lyase enzyme preparation at heat load of 9.2 kW/m^2.

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.

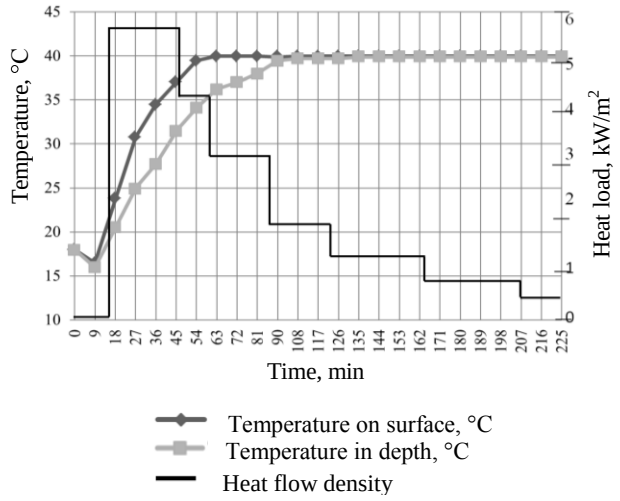


Fig. 14. Sublimation drying curves of the L-phenylalanine ammonia-lyase enzyme preparation at heat load of 5.52 kW/m^2.

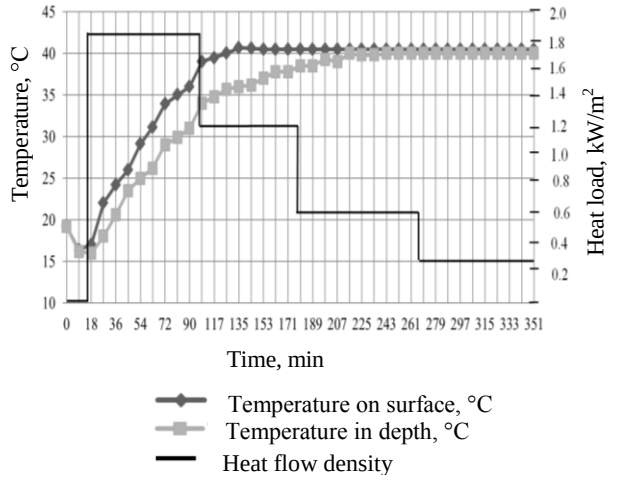


Fig. 15. Sublimation drying curves of the L-phenylalanine ammonia-lyase enzyme preparation at heat load of 1.84 kW/m^2.

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.

REFERENCES

1. Gusarov, D.A., Freeze-drying of biopharmaceutical proteins. *Russ. J. Biopharmaceuticals*, 2010, vol. 2, no. 5, pp. 3–7.
2. Nikitin, E.E., and Zvyagin, I.V., *Zamorazhivanie i vysushivanie biologicheskikh preparatov* (Freezing and Drying of Biological Preparations), Moscow: Kolos, 1971.
3. Lykov, A.V., *Teoriya suchki* (Theory of Drying), Moscow: Energiya, 1968.
4. Babakin, B.S., and Lepikhina, O.E. Sovremennoe sostoyanie i perspektivy razvitiya vakuumnoi sublimatsyonnoi sushki (Modern state and prospectives for development of freeze-drying), *Kholodil'naya tekhnika (Refrigeration Engineering)*, 2005, no. 11, pp. 56–59.
5. Kretov, I.T., Shakhov, S.V., Ryazanov, A.N., et al., Vybor optimal'nogo istochnika nagreva dlya vacuum-sublimatsyonnoi sushki produktov biologicheskogo proiskhozhdeniya (), *Khranenie i pererabotka sel'khoz syr'a (Storage and Processing of Agricultural Raw Materials)*, 2001, no. 1, pp. 12–14.
6. Karpov, A.M., and Ulumiev, A.A. *Sushka produktov mikrobiologicheskogo sinteza* (Drying of Products of Microbiological Synthesis), Moscow: Kolos, 2000.
7. Baldi, C., Gasco, M., and Pattarino, F., Statistical procedures for optimizing the freeze-drying of a model drug in tret-butil alcohol-water mixtures, *Eur. J. Pharm. Biopharm.*, 1994, vol. 40, no. 3, pp. 138–141.
8. Santivarangkna, C, Kulozik, U, and Foerst, P. Effect of carbohydrates on the survival of *Lactobacillus helveticus* during vacuum drying, *Lett. Appl. Microbiol.*, 2006, vol. 42, no. 3, pp. 271–276.
9. Abadias, M., Benabarre, A., Teixido, N., Usall, J., and Vinas, I., Effect of freeze-drying and protectants on viability of the biocontrol yeast *Candida sake*, *Int. J. Food Microbiol.*, 2001, vol. 65, no. 3, pp. 173–182.
10. Champagne, C.P., Gardner, N., Brochu, E., and Beaulieu, Y., A review: the freeze-drying of lactic acid bacteria, *Can. Inst. Food Sci. Technol. J.*, 1991, vol. 24, pp. 118–128.

PROCESSES, EQUIPMENT, AND APPARATUSES OF FOOD INDUSTIRY

INTENSIFICATION OF YEAST BIOMASS CULTURING IN A FILM BIOREACTOR

N.A. Voinov, O.P. Zhukova, and A.N. Nikolaev

Siberian State Technological University,
pr. Mira 82, Krasnoyarsk, 660049 Russia,
phone/fax: 391-227-34-53, e-mail: Voynov@Siberianet.ru

Received March 5, 2013; accepted in revised form April 27, 2013

Abstract: The results of Doctor N.A. Voinov and his colleagues' research aimed at developing gas–liquid film bioreactors are systematized. Fluid dynamics and heat and mass transfer in a liquid film flowing by gravity down a surface with artificial large-scale roughness have been investigated. Relationships based on the plug flow model are suggested for calculating mass transfer in the working zones of the bioreactor. Ways of raising the productivity of the apparatus and reducing the cost of culturing Candida scottii yeast are considered. Closed-loop gas circuit schemes are suggested for the film bioreactor.

Key words: film bioreactor, yeast biomass, heat transfer, mass transfer, artificial roughness

UDC 663.12:57.083.13
DOI 10.12737/2057

INTRODUCTION

Gas–liquid bioreactors are widely used in the food and related industries, including the production of enzyme preparations, baker's yeast, biopolymers, and other microbiological synthesis products. One of the serious drawbacks of the existing industrial bioreactors is their low biomass output capacity, high stirring and concentrating costs, and large mounts of microbiological synthesis products discharged into the environment because of the low intensity of heat transfer. This imposes limits on the composition of te gaseous substrate, generates temperature distribution nonuniformities in the culture liquid, and does not ensure proper purification of the exit gas from metabolism products.

Of the wide variety of the existing fermenters, the most widely employed ones are bubblers with mechanical stirring and liquid circulation in the working space of the apparatus. However, the energy consumption per unit weight of the resulting biomass in stirred bioreactors is higher than in the other types of apparatuses [1] and is 3–4 (kW h)/kg at a comparatively low biomass concentration (Table 1). Although the stirring of the liquid increases the interfacial area owing to the breakup of gas bubbles, the liquid flow remains mainly laminar and the energy consumed does not afford an adequate increase in the rate of oxygen transport in the liquid phase. In addition, heat removal from the reaction zone of the apparatuses is slow and, as a consequence, the culturing process often takes place at a non-optimal temperature.

One way of increasing the output capacity of the gas–liquid bioreactor is by saturating the culture liquid with the gas in a turbulent liquid film. [2–4] flowing down the surface of contact devices. This technique has found application in microorganism culturing methods developed by the authors [5–10].

Table 1. Characteristics of bioreactors

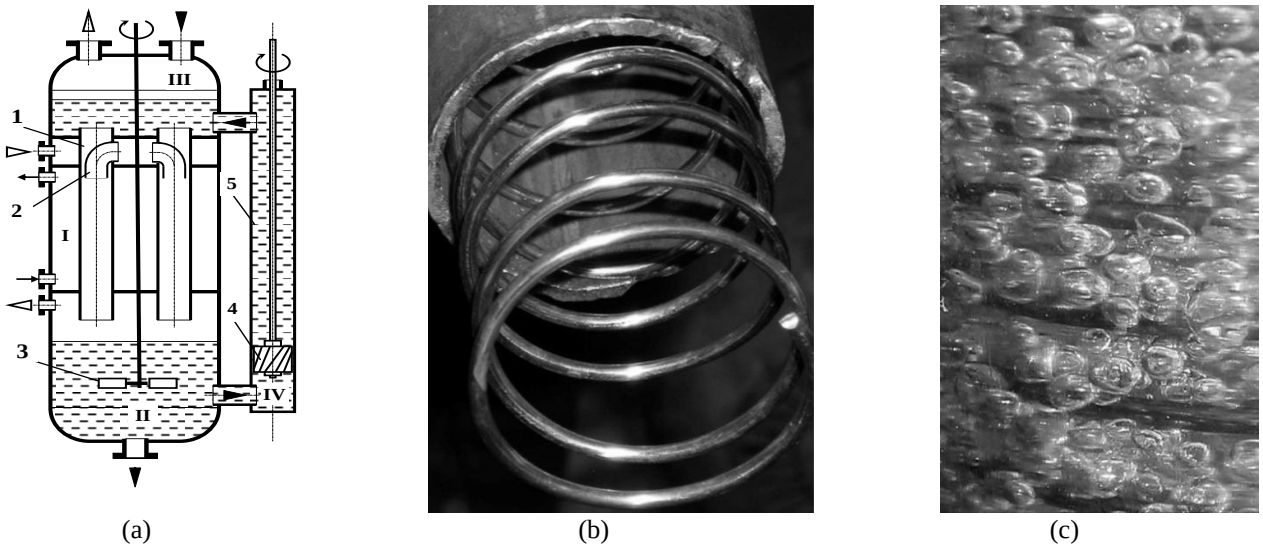
Characteristic	Gas-lift bioreactor	Stirred gas-lift bioreactor	Bioreactor with a suction stirrer	Jet-stirred bioreactor	Film bioreactor
Oxygen transport rate, kg/(m^3 h)	1.1–4.0	4–7	7	0.4–0.7	10
Bulk mass transfer coefficient, h^{-1}	200–450	450–1000	1000	100–150	1500–4000*
Surface mass transfer coefficient, m/s	$(1.3–5) \cdot 10^{-4}$	$(5–6) \cdot 10^{-4}$	$6 \cdot 10^{-4}$	$(0.4–0.7) \cdot 10^{-4}$	$(2–5)^* \cdot 10^{-2}$
Concentration of reducing substances, kg/m^3	8 - 30	30	30	8	100
Specific air consumption, m^3/kg	30–50	34	29–43	10	0–10
Biomass concentration (ADW), kg/m^3	4–10.5	10	10	4	40–80**
Filling factor	0.33	0.7	0.4	0.33	0.8
Specific energy consumption, kW h/kg	0.8–1.75	2–3	3–4	0.4–.5	0.6–1.4
*In the falling liquid film. **Calculated value.					

The introduction of a falling-film section for gas absorption in the culture liquid into the stirred apparatus makes it possible to significantly intensify heat and mass transfer and to reduce specific expenses.

Film bioreactors are next-generation apparatuses, and their introduction into industry is impeded by the poor understanding of the heat transfer processes occur-

ring there. The oxygen transfer rate in the falling liquid film can reach 10 kg/(m^3 h) or over [11], and the surface mass transfer coefficient can be up to (2–5) · 10^{-2} m/s, one order of magnitude larger than in the other types of fermenters. Heat transfer in a turbulent film [12] is also more intensive than in bubblers and gas-lift apparatuses. Furthermore, biomass growth in film bioreactors can be carried out without employing mechanical devices for transporting the components of the gaseous substrate, since the gas is uninvolved in the generation of the phase contact surface and in liquid turbulization. Owing to the high rates of oxygen supply and metabolite removal, film bioreactors are capable of processing concentrated nutrient media, ensure a high product output rate and fine purification of the spent gas from metabolites and substrate drops, maintain a high degree of sterility in the process, and make it possible to organize a closed-loop gas circuit and gas cleaning in the apparatus.

A possible design of the film bioreactor [13–15] is presented in Fig. 1. Four heat transfer zones can be distinguished in the apparatus. These are a liquid film saturation chamber (I), a mechanically stirred chamber (II), a liquid inlet chamber (III), and a flow circuit (IV). Only when the necessary concentration of the gaseous substrate dissolved in the liquid and the necessary temperature are ensured in each of these zones can a high product output capacity be attained. Fluid dynamics and mass transfer in the mechanically stirred chamber II have been comprehensively investigated to date, while mass transfer in the falling liquid film in chamber I needs to be further analyzed.



(a) (b) (c)

Fig. 1. Film bioreactor: (a) reactor design, (b) contact device, and (c) gas bubbles in the falling liquid film; (*1*) tube of the contact device, (*2*) gas pipe, (*3*) stirrer, (*4*) pump, and (*5*) circulation pipe.

DATA SYSTEMATIZATION

Here, we present an analysis of results obtained by N.A. Voinov and his colleagues in their research and development works on film bioreactors. The methods of acquisition and processing of hydrodynamic and heat and mass transfer data for turbulent falling films are described elsewhere [16, 17]. Experiments were carried out on *Candida scottii* yeast and on hydrogen bacteria *Ralstonia eutropha* [18–20].

Fluid dynamics in the film saturation chamber. The highest heat and mass transfer efficiency and the highest liquid throughput capacity are achieved with contact devices whose surface has large-scale artificial helical-rib roughness made from a wire or ribbon [10] (Fig. 1b). In this case, part of the liquid, flowing between roughness coils, is set in combined rotational and translational motion, which favors its uniform distribution along the contact device perimeter without separation and formation of jet flows even on fouled and tilted film-forming surfaces.

The optimum height of the ribs of the helical roughness (wire diameter or ribbon width) is h = 3–5 mm [21]. As the rib height is further increased, there is no significant increase in the saturation intensity because of decrease in the phase contact area. The maximum possible rate of liquid flow through the contact device at its diameter of 45–100 mm and h = 3–4 mm can be estimated using the relationship

$$G = 1{,}2\exp(0{,}04 \cdot d), \quad (1)$$

where G is the highest liquid flow rate allowing film flow (m^3/h) and d is the inner diameter of the contact device tube (mm).

For example, at a contact device diameter of 48 mm, the liquid flow rate ensuring the formation of an annular falling film is 2 to 8 m^3/h. At a lower liquid flow rate, the surface is incompletely wetted; at a higher flow rate, there will be a decrease in the phase contact area. An important design feature of the contact device is that the upper coils of the roughness helix should be placed in the annular gap between the gas pipe and the tube surface in order to set the liquid in rotational and translational motion and to prevent its breaking away from the rib edges upon its running on the roughness ribs.

Contact devices may be made from ribbon coils with a deflected side edge [10] or from a porous body [15]. In the former case, the metal consumption is reduced; in

the latter case, the saturation of the liquid with the gaseous substrate is markedly intensified.

The thickness of the gas–liquid film flowing down the surface of a contact device with helical roughness is several times larger than the thickness of the film on a hydraulically smooth wall and can reach a value of 25 mm or above [22, 23] (Fig. 2). The liquid flow in the former case is stable in a wide range of flow rtes (Reynolds number Re = $4G/\nu\pi d$ = 20 000–100 000, where G is the liquid flow rate (m^3/s) and ν is the kinematic viscosity of the yeast suspension (m^2/s).

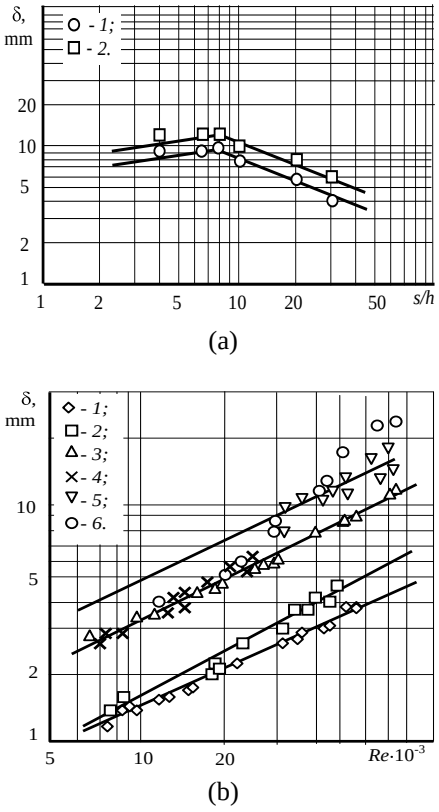


Fig. 2. Thickness of the falling liquid film as a function of the (a) roughness parameter and (b) Reynolds number at d = 51 mm, $\nu = 1 \cdot 10^{-6}$ m^2/s, and a contact device tube length of l = 1.6 m. (a) h = 5.5 mm; Re = (*1*) 30 000 and (*2*) 45 000. (b) s/h = 8; (*1*, *2*) film flowing down the outer surface of the contact device tube, h = 0; (*3*–*7*) film flowing down the inner surface, h = (*3*) 1.85, (*4*) 3.0, (*5*) 5.5, and (*6*) 6.0 mm.

Experiments demonstrated that, as the roughness rib height and the dynamic viscosity of the yeast suspension are increased, the falling liquid film on the contact device surface becomes thicker. The largest thickness value is observed at a roughness parameter of s/h = 4–8 (Fig. 2a), where s is the roughness coil spacing. Surfactants do not exert any significant effect on the thickness of the liquid film.

The thickness of the gas–liquid film increases with the distance traveled by the film and becomes constant at $l \approx 1.6$ m. It is suggested that the liquid film thickness in the valley of the helical roughness, δ_v (Fig. 3), be calculated using the following relationship [10]:

$$\delta\left(1 \pm \frac{\delta}{2R}\right) = \left(1 \pm \frac{h}{2R}\right)\frac{hs}{s+e} + \left\{\frac{\partial^2}{\rho^2 g}\,\frac{1 \pm h/R}{}\left[\frac{\lambda e}{8}\,\frac{}{s+e} + \frac{s}{4\,\pi^{1/2}\sigma_1}\,\frac{}{s+e}\right]\right\}^{1/3}, \quad (2)$$

where ρ is the density of the gas–liquid mixture (kg/m^3), e is the width of a roughness rib (m), λ is the friction factor of the liquid film on the smooth wall of the tube, $\Gamma = G/\pi d$ is the mass irrigation density (kg/(s m), R is the tube radius (m), $\sigma_1 = 8$ is an empirical correction, the sign "+" refers to a film flowing down the outer surface of the tube, and the sign "–" refers to a film flowing down the inner surface of the tube.

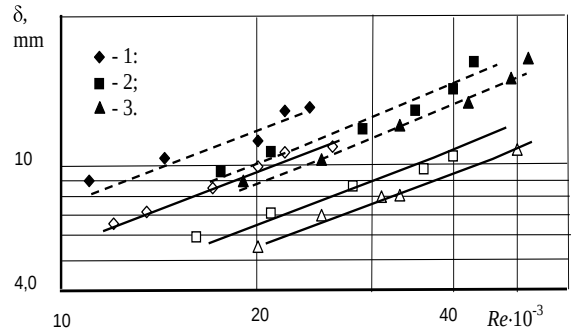


Fig. 3. Thickness of the yeast suspension film versus Reynolds number at d = 51 mm, s/h = 6, and h = 3 mm. Experimental data points: ν = (*1*) $1.2 \cdot 10^{-6}$, (*2*) $0.66 \cdot 10^{-6}$, and (3) $0.55 \cdot 10^{-6}$ m^2/s. The solid and dashed lines represent data pertaining to roughness valleys and roughness ribs, respectively.

The density of the gas–liquid mixture in Eq. (2) is given by

$$\rho = \rho_1(1 - \varphi) + \rho_g \varphi,$$

where φ is the gas content and ρ_g and ρ_l are the gas and liquid densities.

The friction factor is calculated using Eq. (3) and experimental data (Fig. 4) [10]:

$$\lambda_r = \frac{8\rho^2 g h^3}{\Gamma^2}\left\{\left(1 \pm \frac{h}{2R}\right)\frac{s}{s+e} + \frac{\Gamma^{2/3}}{h\rho^{2/3}g^{1/3}}\,\frac{1 \pm h/R}{}^{1/3} \times \left[\frac{\lambda e}{8}\,\frac{}{s+e} + \frac{s}{4\sqrt{\pi\sigma}}\,\frac{}{s+e}\right]^{1/3}\right\}^3, \quad (3)$$

The kinematic viscosity of the *Candida scottii* yeast suspension as a function of the biomass concentration (x = 10–100 kg/m^3 at 38°C) was calculated as follows [10]:

$$\nu = 7.397 \cdot 10^{-7} + 2.113 \cdot 10^{-8} x, \quad (4)$$

where x is the microorganism concentration (kg/m^3).

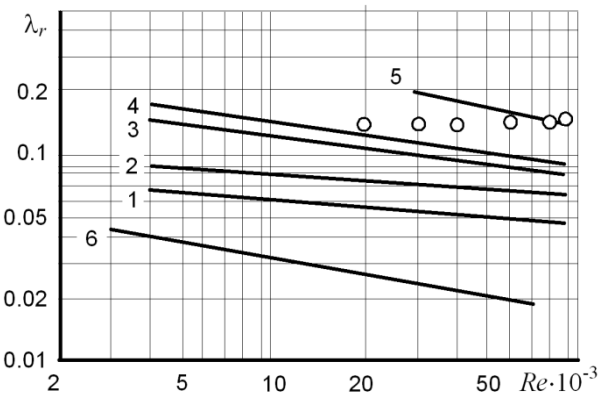


Fig. 4. Friction factor versus Reynolds number for a liquid film flowing down the tube surface at d = 30 mm, l = 2.5 m, s/h = 10, and $\nu = 1 \times 10^{-6}$ m^2/s. The lines represent the data calculated via Eq. (3) at h = (*1*) 0.1 (*2*) 0.18, (*3*) = 0.33, (*4*) 0.45, and (*5*) 1.0 mm; (*6*) data calculated using the $\lambda = 0.3164/Re^{0.25}$ equation. The points represent experimental data [24].

When the microorganism concentration in the culture liquid is above 100 kg/m^3 the kinematic viscosity increases dramatically because of the structuring of the suspension, confirming the existence of an upper limit for the Newtonian flow of suspensions.

The kinematic viscosity of the yeast suspension at a culturing temperature of 30°C is given by the relationship

$$\nu = (0.0197x + 0.793)\cdot 10^{-6}. \qquad (5)$$

The density of the yeast suspension at 30°C and x = 100 kg/m^3 is 1025 kg/m^3, and that at x = 200 kg/m^3 is 1065 kg/m^3. The dependence of the density of the medium on the bacterial biomass concentration under the biosynthesis conditions can be represented as $\rho = 995.6\exp(0.0002\cdot x)$.

As the culture liquid flows down the surface with large-scale helical roughness, gas bubbles nucleate in roughness valleys (Fig. 1c), and this leads to the formation of a gas–liquid mixture. This gas bubble nucleation is due to boundary layer separation taking place when the liquid flows from roughness ribs, which generates circulation vortices in the valleys [25]. Once a certain static pressure drop between the vortex and the liquid film surface is reached, which depends on the surface tension and thickness of the film, the gas penetrates into the hollow of the vortex, and this causes the formation of gas bubbles and their buildup in the liquid.

With the optimal arrangement of the coils of the helical roughness, i.e., at s/h = 4–8, the roughness valleys are filled with gas bubbles to the greatest extent and the bubble diameter is comparable to the height of the roughness rib and is 1.5–4 mm. The gas content of the liquid increases as the height of the rib of the regular helical roughness is increased to h = 3 mm and then remains constant, φ = 0.45. The increase in the gas content as a result of an increasing roughness rib height is due to the change in the vortex scale.The interfacial area of the liquid film flowing down the helix-roughened surface was 700–1000 m^2/m^3, while the interfacial area in apparatuses with a turbine-type agitator at its specific power of 0.5–4 kW/m^3 is much smaller [1, 26, 27] and is a = 75–200 m^2/m^3.

Heat and mass transfer in the film saturation chamber. Mass transfer in a turbulent liquid film has been the subject of numerous works, including studies dealing with contact devices with a rough surface [28–30].

Figure 5 plots the effective mass transfer coefficient in the falling liquid film versus Re as calculated without taking into account the surface area of the gas bubbles. It was established that the highest saturation efficiency is attained at a roughness parameter of s/h = 4–8.

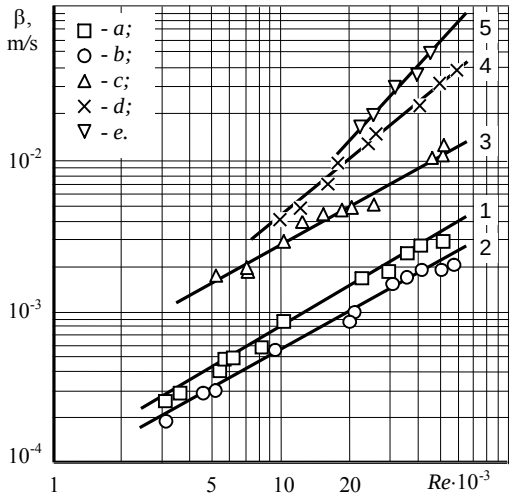


Fig. 5. Effective mass transfer coefficient versus Reynolds number for a water film flowing down (lines *1*, *2*) smooth and (lines *3*–*5*) rough vertical tube surfaces at
t = 20°C. Experimental data points: (a) inner surface (d = 27 mm, l = 2 m); (b) outer surface (d = 30 mm, l = 2 m); (c–e) internal surface (d = 51 mm, l = 1.6 m, s/h = 10), h = (c) 1.85, (d) 3.0, and (e) 5.0 m.

At a dimensionless roughness rib height of , the effective mass transfer coefficient can be calculated using the following relationship [29]:

$$Sh^* = 6.45\cdot 10^{-4}(\mathrm{Re}^*)^{1.505} Sc^{0.5}, \qquad (6)$$

for h^+ = 150 – 1600,

$$Sh^* = 6.45\cdot 10^{-4}(\mathrm{Re}^*)^{1.505} Sc^{0.5}\left(\frac{h^+}{150}\right), \qquad (7)$$

Here, $\mathrm{Re}^* = 4\delta u^*/\nu$, $\mathrm{Sh}^* = \beta\delta/D_l$, and $\mathrm{Sc} = \nu/D_l$.

The "dynamic" liquid velocity on the rough wall of the tube for a film flowing by gravity is calculated via the formula

$$u^* = \left(\frac{\lambda_r}{8}\right)^{0,5} \frac{\Gamma}{(1 \pm \delta/(2R))\rho\delta}, \qquad (8)$$

where λ_r is the friction factor for the liquid (Fig. 4).

tween the gas and the surface of the falling film and as interaction between the gas bubbles and the liquid. With this approach, the coefficient of mass transfer between the bubbles of the gaseous substrate and the liquid is $(0.8–2) \cdot 10^{-3}$ m/s and mass transfer coefficient in the liquid film bulk is $(2–8) \cdot 10^{-3}$ m/s.

Aeration of a falling liquid film thorough the porous surface of a tubular insert makes it possible to intensify liquid saturation with the gas by a factor of up to 2 [10]. The presence of a surfactant in the fermentation medium reduces the mass transfer coefficient [31] (Fig. 6).

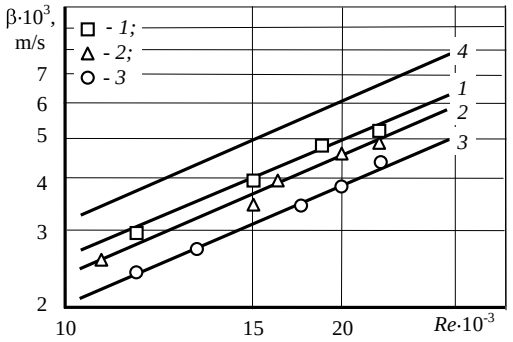


Fig. 6. Mass transfer coefficient versus Reynolds number for the gravity flow of a fermentation medium film along the tube surface with helical roughness at d = 51 mm, l = 1.24 m, h = 2 mm, and s/h = 8. (*1–3*) experimental data points: σ = (*1*) $52 \cdot 10^{-3}$, (*2*) $42 \cdot 10^{-3}$, and (*3*) $34 \cdot 10^{-3}$ N/m. (*4*) Experimental data for distilled water.

The surface tension values measured for the fermentation medium are plotted in Fig. 7. There are two characteristic regions of the reducing substance (S) concentration effect on the surface tension σ. The surface tension of the fermentation medium is much higher than the surface tension of the culture liquid.

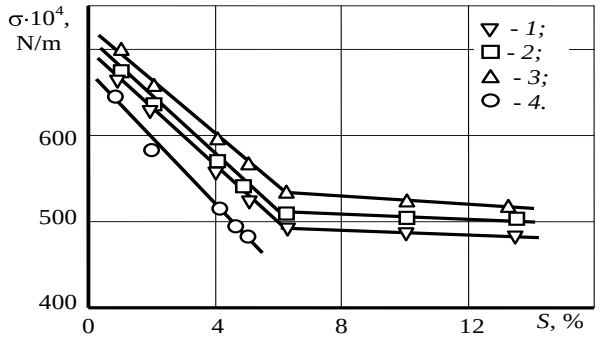


Fig. 7. Surface tension of the fermentation medium versus the concentration of reducing substances of molasses. (1–4) Experimental data points: t = (*1*) 45, (*2*) 30, and (*3*) 10°C; (4) hydrolysate from wheat grains at t = 20°C.

The surface tension of a bacterial suspension grown in Schlegel's medium is

$\sigma = (74–79) \cdot 10^{-3}$ N/m in the concentration range x = 1–80 kg/m^3 at 30°C.

As water evaporates from the surface of the falling liquid film during culturing, the equilibrium gas concentration decreases according to the following relationships because of the change of the partial pressure at the interface [31] (Fig. 8):

for the smooth surface of the contact device tubes,

$$\frac{c_g}{c^*} = \exp(-0.06 \cdot R), \quad (9)$$

for the surface with helical roughness,

$$\frac{c_g}{c^*} = \exp(-0.014 \cdot R), \quad (10)$$

Here, c^* is the equilibrium oxygen concentration when there is no vapor outflow, c_g is the equilibrium oxygen concentration in the case of vapor outflow taking place, and R is the specific water vapor flow rate (kg/(m^2 h)).

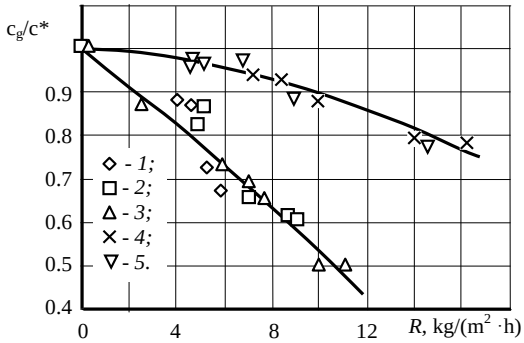


Fig. 8. Ratio of the oxygen concentrations in the liquid at the interface versus the specific flow rate of evaporated water for the liquid film flowing downwards by gravity. Experimental data points: (*1–3*) smooth tube surface, t_g = 25°C, t_l = (*1*) 40, (2) 50, and (3) 60°C; (*4, 5*) rough surface, t_g = 25°C, t_l = (*4*) 50 and (*5*) 40°C.

Measurements demonstrated that the solubility of oxygen in the fermentation media used is approximately 10% lower than in distilled water.

The presence of microorganisms showing a certain respiratory activity in the liquid reduces the concentration of dissolved oxygen in the falling liquid film at the outlet of the contact device, and it is necessary to take into consideration this circumstance when designing a bioreactor.

Under the assumption that the contact device (zone I in Fig. 1a) is a plug flow reactor, the mass balance equation for the dissolved gas concentration C in the falling culture liquid film with thickness δ can be written as

$$u\delta \frac{dc}{dl} = \beta(c^* - c) - qx\delta, \quad (11)$$

where u is the mean velocity of the liquid film (m/s), l is the tube length (m), x is the yeast concentration in the liquid (kg/m^3), q is the respiratory activity of the yeast (kg/(kg s)), c^* is the equilibrium gas concentration in the liquid (kg/m^3), and β is the surface mass transfer coefficient (m/s).

At a constant gas partial pressure in the contact zone, we will then obtain

$$c = c^* - \frac{qx\delta}{\beta} + \left(\frac{qx\delta}{\beta} + c_o - c^*\right)\exp\left(-\frac{\beta l}{u\delta}\right), \quad (12)$$

where c_o is the concentration of dissolved gas in the liquid at the chamber inlet (kg//m^3).

Equation (12) provides means to calculate the dissolved gas concentration in the liquid flowing downwards along the tubular insert. According to the data obtained (Fig. 9), the highest intensity of oxygen supply to the culture liquid is observed at a length of 1.5–2.5 m along the tube. As the microorganism concentration in the liquid is increased, the amount of oxygen in the film at the outlet decreases. At x = 100 kg/m^3 (Fig. 9), the oxygen concentration in the liquid film practically does not increase; that is, the rate of oxygen supply to the liquid is equal to the rate of oxygen consumption by the microorganisms.

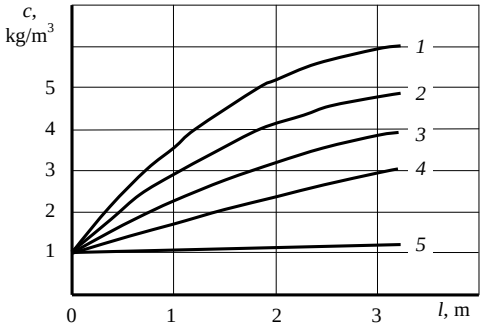


Fig. 9. Variation of the oxygen concentration in the yeast suspension film along the length of the tubular insert at d = 51 mm, h = 3 mm, s/h = 10, Re = 50 000, t = 38°C, c^* = 6 · 10^{-3} kg/m^3, c_0 = 0.1c^*, and q = 4.17 · 10^{-5} kg/(kg s). The lines represent the data calculated for different yeast biomass concentrations: x = (*1*) 0.1, (*2*) 10, (*3*) 20, (*4*) 50, and (*5*) 100 kg/m^3.

Figure 10 presents the results of investigation of heat transfer in the liquid film flowing down the surface with artificial roughness for various rib heights [32]. The largest value of the heat transfer coefficient is observed at a rib height of 0.13 mm and a dimensionless parameter of $h^+ > 29$. The heat transfer coefficient decreases with an increasing rib height because the gas content increases and the heat conductivity decreases according to the relationship

$$\lambda_{mix} = \lambda_l(1-\varphi) + \lambda_g\varphi, \quad (13)$$

where λ_{mix}, λ_l, and λ_g are the heat conductivities of the gas–liquid mixture, liquid, and gas, respectively (W/(m K)).

The following equation was suggested for calculating the heat transfer coefficient in a liquid film flowing down a surface with large-scale artificial roughness [32, 33]:

$$Nu^* = \frac{\alpha\delta_v}{\lambda_{mix}} = 1.2\cdot 10^{-2}\,\mathrm{Re}^{0.8}\,\mathrm{Pr}^{0.6}, \quad (14)$$

where Pr = ν/a is the Prandtl number at the temperature of the liquid.

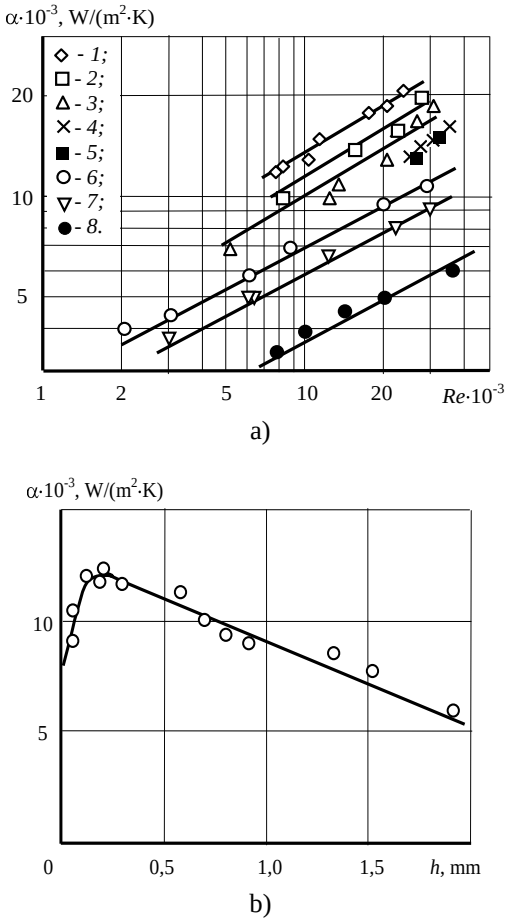


Fig. 10. Heat transfer coefficient α versus (a) the Reynolds number of the liquid flowing by gravity and (b) the rib height of the helical roughness: (a) d = 30 mm; l = 1.9 m; Pr = 4,5; s/h = 10; film on the outer surface of the tube; h = (*1*) 0.13 (*2*) 0.3 (*3*) 0,7, (*4*) 0.85, (*5*) 1.0 , and (*6*) 1.5 mm; (*7*, *8*) film on the inner surface of the tube, d = 51 mm, l = 2.3 m, h = (*7*) 2.0 and (*8*) 3.0 mm; (b) d = 30 mm, s/h = 10, Pr = 4–5, l = 1.9 m, Re = 12 000.

Mass transfer in the mechanically stirred chamber. When the axial velocity of the yeast suspension in the chamber is 0.008 m/s, the microorganisms are uniformly distributed in the liquid [10]. At a lower velocity, yeast flotation takes place because of the presence of gas bubbles in the bulk of the culture liquid and, therefore, the liquid should be mechanically stirred.

Two mass transfer zones can be distinguished in the working space of the chamber. These are the jet aeration zone and the mechanical stirring zone. As the liquid flows off the contact devices, the resulting jets with the gas entrained by them plunge into the liquid in the chamber. The greatest degree of mass transfer intensification in this case is observed when the jets travel a distance of l = 200 mm. As this distance is further increased, the mass transfer coefficient decreases because the liquid breaks up into drops and, as a consequence, its momentum diminishes. The values of the bulk mass transfer coefficient in jet aeration are plotted in Fig. 11.

The following relationship is suggested for calculating the bulk mass transfer coefficient in jet aeration [10]:

$$\beta_v = 6 \cdot 10^{-8} \cdot \mathrm{Re}_f \mathrm{Sc}^{0.5}, \qquad (15)$$

where $\mathrm{Re}_f = 2\delta u/\nu$ is the Reynolds number and Sc is the Schmidt number.

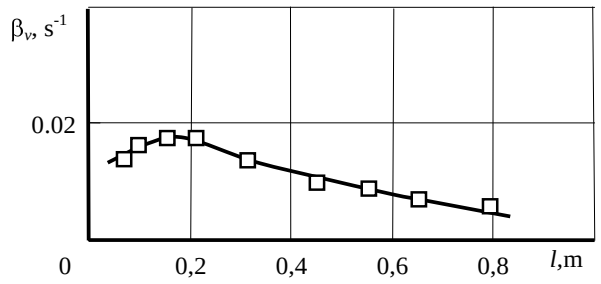


Fig. 11. Bulk mass transfer coefficient in the jet aeration of water as a function of the distance traveled by the jet at d = 51 mm, t = 20°C, and an outflow velocity of 1.2 m/s.

The height of the jet-aerated zone in the bulk of the culture liquid does not exceed 0.35 m, and its diameter is 0.2 m.

Two hydrodynamic regimes are observed in the mechanically stirred mass transfer zone [26, 34] (Fig. 12). The experimental values of the bulk mass transfer coefficient in apparatuses with a turbine or blade stirrer at $N(1 - \varphi)/V < 10$ in relation to the rotational speed of the stirrer, gas flow rate, and the liquid column height in the chamber [26] are presented in Fig. 12a, and the same data for $N(1 - \varphi)/V > 10$ are plotted in Fig. 12b, where N is the power spent on stirring (W) and V is the volume of the chamber (m^3).

The best agreement between the calculated and experimental values of the surface mass transfer coefficient β is provided by the following equation [35]:

$$\beta = 0.33\left(\frac{n d_{st} d_s}{\nu}\right)^{0.6} Sc^{\frac{1}{2}}\left(\frac{D_1}{d_s}\right), \qquad (16)$$

where d_{st} is the diameter of the turbine-type stirrer (m), d_s is the surface-mean diameter(m), n is the rotational speed of the stirrer (s^{-1}, and D_l is the diffusion coefficient (m^2/s).

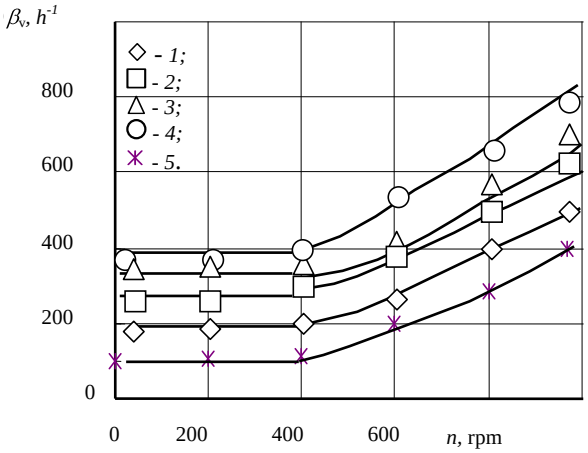
The gain in the mass transfer coefficient achieved by mounting a second turbine-type stirrer on the same shaft was 30% and was due to the further breakup of gas bubbles in the liquid.

The bubble diameter was calculated using the following formula:

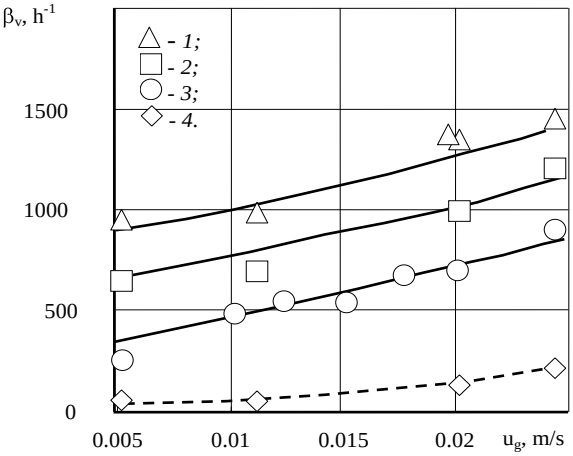
$$d_s = 0.8\left[\frac{\sigma^{0.6}}{\left(\frac{N}{V}\right)^{0.4} \rho_1^{0.2}}\right], \qquad (17)$$

The mass balance equation for the variation of the dissolved gas concentration C along the height h of the aerated layer can be written as

$$u\frac{dc}{dh} = \beta_v(c^* - c) - qx, \qquad (18)$$



(a)



(b)

Fig. 12. Bulk mass transfer coefficient as a function of (a) the rotational speed of the stirrer and (b) the flow rate average velocity u_g in the case of a bubbler installed. (a) Blade stirrer, apparatus diameter of D = 0.38 m, d_{st} = 0.11 m; experimental data points: (*1*) u_g = 0.018 m/s, H/D = 1; (*2*) u_g = 0.03 m/s, H/D = 1; (*3*) u_g = 0.04 m/s, H/D = 1; (*4*) u_g = 0.04 m/s, H/D = 0,5; (*5*) turbine-type stirrer, d_{st} = 0.075 m, D = 0.216 m, u_g = 0.01 m/s, H/D = 1, where H is the height of the liquid column. (b) Turbine-type stirrer, D = 0.4 m; experimental data points: (*1*) two stirrers on one shaft, d_{st} = 0.150 m; (*2*) one stirrer, d_{st} = 150 mm; (*3*) one stirrer, d_{st} = 80 mm, D = 160 mm; (*4*) bubbler without a stirrer.

With the boundary conditions $h = 0$, $c(h) = c_{up}$, we obtain

$$c = c^* - \frac{qx}{\beta_v} + \left(\frac{qx}{\beta_v} + c_{in} - c^*\right)\exp\left(\frac{\beta_v h}{u}\right), \qquad (19)$$

where c_{in} is the gas concentration in the upper layer of the liquid in the bioreactor, u is the mean velocity of the culture liquid in the working zone, β_v is the bulk mass transfer coefficient (s^{-1}), and h is the height of the liquid layer in chamber II (m).

Relationship (20) provides means to calculate the gas concentration in the liquid along the height of the aerated layer in the working space of the bioreactor in

the case of microorganisms uniformly distributed in the apparatus (Fig. 13).

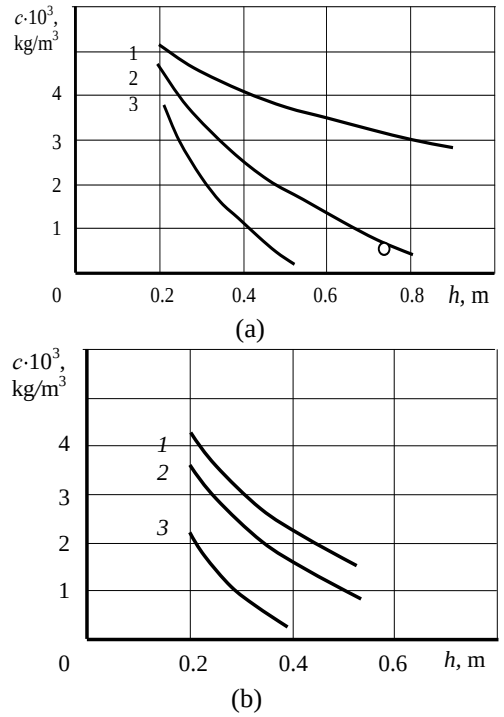


Fig. 13. Oxygen concentration profiles along the height of the aerated layer of the culture liquid in the bioreactor: (a) u = 0.1 m/s, x = 30.6 kg/m^3, q = 0.16 kg/(kg h), β_v = (*1*) 0.55, (*2*) 0.15, and (*3*) 0.001 s^{-1}; (b) x = 44 kg/m^3, q = 0.16 kg O_2/(kg h), β_v = 0.15 s^{-1}, u = (*1*) 0.15, (*2*) 0.1, and (*3*) 0,05 m/s.

Mass transfer in the flow circuit of the bioreactor. The variation of the dissolved gas concentration along the length l of the flow circuit IV and in chamber III can be calculated using the following relationship (20):

$$u\frac{dc}{dl} = -qx\,, \qquad (20)$$

Integration of this relationship subject to the boundary conditions $l = 0$, $c = c_c$ yields

$$c = c_c - \frac{qxl}{u}\,, \qquad (21)$$

where c_c is the gas concentration in the culture liquid at the inlet of the flow circuit.

According to the aforesaid, in the calculation of bioreactor parameters the concentration of dissolved gas in the culture liquid at the outlet of the film flow chamber is determined from relationship (12). The concentration of the dissolved gaseous substrate can be varied both by regulating the liquid flow rate in the flow circuit and, in some case, by regulating the gas phase velocity in the contact devices. The mass transfer coefficient data (Figs. 5, 6) suggest that the concentration of microorganisms in the film saturation chamber (compared to the other zones of the bioreactor) is not limited by the gas substrate. Conversely, it ensures a dissolved gas reserve that is then consumed in chamber I.

Table 2. Calculated characteristics of bioreactors

Characteristic	Airlift S = 1.5%	Film S = 3%	Film S = 7%
Output capacity, t/day	6	6	6
Amount of metal required, t	46.6	20	18
Apparatus diameter and height, m	7.4 ×14.2	5.5 × 10	3.5 × 10
Specific energy input in culturing, kW/kg	1.2	0.8	0.9
Air flow rate, m^3/h	12 500	0–3000	0–3000
Fresh water flow rate, m^3/h	44	0	0
Spent liquid (discharge) flow rate, m^3/h	69.0	12.8	8.2
Electricity consumption, kW h	428	232	200

Calculations demonstrated that, as the concentration of reducing substances in the nutrient wort increases in the S = 1–10% range at a given output rate, the necessary volume of the bioreactor decreases and the concentration of microorganisms in the culture liquid increases with an insignificant increase in energy consumption for gaseous substrate transport (Fig. 14).

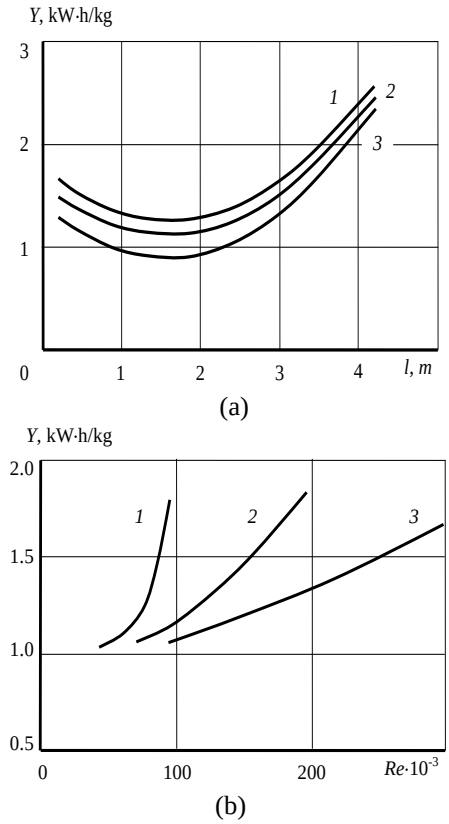


Fig. 14. Specific energy consumption versus (a) the contact devices length l and (b) the Reynolds number of the liquid: (a) d = 80 mm, h = 4 mm, s/h = 6, Re = 170 000, S = (*1*) 5, (*2*) 3, and (*3*) 1%; (b) l = 1.5 m, S = 3%, d = (*1*) 50, (*2*) 80, and (*3*) 100 mm.

Raising the gas pressure in the bioreactor does not lead to any significant change in the energy consumption in the process. The energy input in gas compression is compensated for by the increase in the equilibrium oxygen concentration in the liquid and by the decrease in the power consumed by the circulation pump. The circulation ratio and, accordingly, energy consumption can be reduced by raising the partial pressure of the saturating gas in the contact zone and by installing several film saturation chambers along the apparatus height. It is clear from the data presented in Table 3 that film bioreactors are capable of processing concentrated media at low gaseous substrate and spent liquid flow rates and high microorganism concentrations. They have moderate dimensions and need a comparatively low energy input for their operation.

The film bioreactor provided a basis for designing culturing processes involving a closed-loop gas circuit and gas purification [36]. This is a step forward in developing environmentally friendly microbiological synthesis technologies that rule out continuous air consumption and gas emission into the atmosphere.

The basic principle of developing an environmentally friendly technology is to organize a closed-loop gas circuit. This principle can be implemented by removing the metabolism products released in the reactor and by returning the purified gas into the reactor after the addition of the necessary amount of the required component. Carbon dioxide released by *Candida scottii* can be efficiently removed from the gas by standard chemical or physical sorption methods. In chemical sorption, the reaction products are neutralized or processed. Physical sorption of carbon dioxide involves regeneration of the sorbent (e.g., 20% monoethanolamine solution) and production of carbon dioxide for the food industry.

The process flowsheet involving a closed-loop gas circuit in oxygen production from atmospheric air by bringing it into contact with a neutral liquid is presented in Fig. 15. Atmospheric air is directed to absorber *4*, where it is brought into contact with a neutral liquid having a high oxygen absorption capacity. The oxygen-saturated liquid enters degasser *3*, where oxygen is liberated and is then directed to bioreactor *1*. The gas containing metabolism products flows from the bioreactor to absorber *2* for cleaning to come into contact with the absorbent. The latter is then directed to the regeneration unit, and the clean gas is returned to the bioreactor. This design rules out contact between atmospheric air and the microorganisms, preventing air pollution. The introduction of an oxygen carrier into the neutral liquid will eliminate the problems associated with oxygen recovery from the culture liquid and will reduce the energy required for liquid circulation.

The setup ensuring a high degree of oxygen recovery from air is schematized in Fig. 16. Here, the goal is achieved by removing carbon dioxide, which hampers microorganism growth, from the culture liquid in degasser *2* followed by gas cleaning in absorber *3*. If air is passed through semipermeable membranes in apparatus 4, a decrease in the air flow rate will be achieved owing to the increase in the oxygen concentration in the air. This scheme can be recommended for improving the existing industrial bioreactors by organizing a closed-loop air circuit. This will markedly reduce the flow rate of the air leaving the apparatus and lower expenditures will be required for its cleaning.

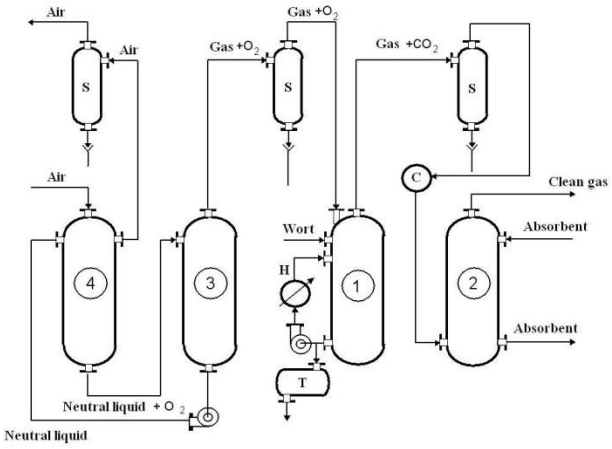


Fig. 15. Growth of microorganisms using a neutral liquid: (*1*) film bioreactor, (*2*, *4*) absorbers, (*3*) desorber; H = heat exchanger, S = separator, T = tank, C = compressor.

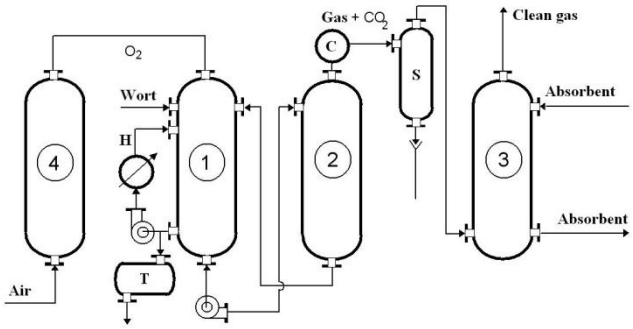


Fig. 16. Growth of microorganisms with a high degree of oxygen recovery from air: (*1*) bioreactor, (*2*) degasser, (*3*) absorber, (4) membrane module; H = heat exchanger, S = separator, T = tank, C = compressor.

Of special interest is the combined culturing of microorganisms consuming oxygen and releasing carbon dioxide together with microorganisms consuming carbon dioxide and releasing oxygen [6], for example, simultaneous production of yeast biomass and chlorella, which is usable as a feed additive. The combined technology provides means to organize environmentally friendly, nonwaste manufacturing of microbiological synthesis products that employs a substantially smaller amount of equipment and needing a much smaller capital input and operating expenses.

CONCLUSIONS

The main advantages of the film bioreactor are that they afford a high biomass concentration in the culture liquid and obviate the need for gaseous substrate transport devices. This makes it possible to organize a closed-loop gas circuit and efficient gas cleaning. The results presented here provide a basis for designing bioreactors.

REFERENCES

1. Kafarov, V.V., Vinarov, A.Yu., and Gordeev, L.S., *Modelirovanie biokhimicheskikh reaktorov* (Modeling of Biochemical Reactors), Moscow: Lesnaya Promyshlennost', 1979.
2. Henstook, W.H. and Hanratty, T.J., *AIChE Journal*, 1979, vol. 25, no. 1, pp.122–131.
3. Hubbard, G.L., Mills, A.F., and Chung, D.K., *Journal of Heat Transfer*, 1976, vol. 98, pp. 319–320.
4. Hewitt, G. and Hall-Taylor, N., *Annular Two-Phase Flow*, Oxford: Pergamon, 1970.
5. USSR Inventor's Certificate no. 1089117, *Byulleten' izobretenii (Inventions bulletin),* 1984, no 16.
6. USSR Inventor's Certificate no. 1655980, *Byulleten' izobretenii (Inventions bulletin),* 1991, no. 22.
7. USSR Inventor's Certificate no. 1507786, *Byulleten' izobretenii (Inventions bulletin),* 1989, no. 34.
8. USSR Inventor's Certificate no. 1717627, *Byulleten' izobretenii (Inventions bulletin),* 1992, no. 9.
9. USSR Inventor's Certificate no. 1717628, *Byulleten' izobretenii (Inventions bulletin),* 1992, no. 9.
10. Voinov, N.A., Sugak, E.V., Nikolaev, N.A., and Voronin, S.M., *Plenochnye bioreaktory* (Film Bioreactors),Krasnoyarsk: BORGES, 2001.
11. Voinov, N.A., Gurulev, K.V., and Volova, T.G., *Biotechnology in Russia*, 2005, no. 3, pp. 98–107.
12. Voinov, N.A. and Nikolaev, A.N., *Teplos"em pri plenochnom techenii zhidkosti* (Heat Transfer in Liquid Film Flow), Kazan: Otechestvo, 2011.
13. Voinov, N.A. and Nikolaev, N.A., *Plenochnye trubchatye gazo-zhidkostnye reaktory* (Tubular Gas–Liquid Film Reactors), Kazan: Otechestvo, 2008.
14. RF Patent 2012593, *Byulleten' izobretenii (Inventions bulletin)*, 1994, no. 9.
15. RF Patent 22211038, *Byulleten' izobretenii (Inventions bulletin),* 2004, no. 1.
16. Nikolaev, A.M., Voinov, N.A., and Nikolaev, N.A., *Theoretical Foundations of Chemical Engineering*, 2001, vol. 35, no. 2, pp. 196–198.
17. Markov, V.A., Voinov, N.A., and Nikolaev, N.A., *Teoreticheskie osnovy khimicheskoi tekhnologii* (*Theoretical foundations of chemical engineering)*, 1990, vol. 24, no. 4, pp. 442–449.
18. Nicolaev N.A., Voinov, N.A., Markov, U.A., *Acta Biotechnologica*, 1991, no. 3, pp. 205–210.
19. Nikolaev, A.N. and Voinov, N.A., *Biotekhnologiya (Biotechnology)*, 2009, no. 5, pp. 74–79.
20. Volova, T.G. and Voinov, N.A., *Applied Biochemistry and Microbiology*, 2004, vol. 40, no. 3, pp. 249–252.
21. Voinov, N.A., Nikolaev, A.N., and Voinova, O.N., *Khimiya rastitel'nogo syr'ya (Vegetable raw materials chemistry)*, 2009, no. 4, pp. 183–193.
22. Voinov, N.A., Konovalov, N.M., and Nicolaev N.A., *Teoreticheskie osnovy khimicheskoi tekhnologii* (*Theoretical foundations of chemical engineering)*, 1993, no. 6, pp. 638–641.
23. Davies, J.T., *AIChE Journal*, 1972, vol. 18, no. 1, pp. 169–173
24. Hisashi, M., Yasushi, K., Toshiyki, H., and Tatsuo, N., *Kaganu Kogaku Ronbunshu*, 1991, vol.2, pp. 308–396.
25. Chang, P., *Control of Flow Separation*, Washington, DC: Hemisphere, 1976.
26. Voinov, N.A. and Volova, T.G., *Khimicheskaya promyshlennost' (Chemical industry),* 2007, vol. 84, no. 3, pp. 145–150
27. Keitel, G. and Onken, U., *German Chemical Engineer*, 1981, vol. 4, pp. 250–258.
28. Markov, V.A., Voinov, N.A., and Nikolaev, N.A., *Theoretical Foundations of Chemical Engineering*, 1991, vol. 24, no. 4, pp. 292–298.
29. Konovalov, N.M., Voinov, N.A., Markov, V.A., and Nikolaev, N.A., *Teoreticheskie osnovy khimicheskoi tekhnologii* (*Theoretical foundations of chemical engineering)*, 1993, vol. 27, no. 3, pp. 309–314.
30. Chung L.K. and Mills A.F., *Letters in Heat and Mass Transfer*, 1974, vol. 1, p. 43.
31. Voinov, N.A., Nikolaev, N.A., Eremenko, N.A., and Karpeza, A.G., *Khimiya rastitel'nogo syr'ya (Vegetable raw materials chemistry)*, 2006, no. 2, pp. 51–60.
32. Voĭnov, N.A., Voĭnova, O.N., and Sugak, E.V., *Thermal Engineering*, 2004, vol. 51, no. 3, pp. 211–215.
33. Voinov, N.A., Zhukova, O.P., and Nikolaev, A.N., *Theoretical Foundations of Chemical Engineering*, 2012, Vol. 46, no. 4, pp. 432–440.
34. Miller, D.N., *AIChE Journal*, 1974, vol. 20, no. 3, pp. 445–453.
35. Strenk, F., *Peremeshivanie i apparaty s meshalkami* (Stirring and Stirred Apparatuses), Leningrad: Khimiya, 1975.
36. Nikolaev, N.A., Voinov, N.A., Markov, V.A., and Gavrilov, A.V., *Biotekhnologiya (Biotechnology)*, 1993, no. 3, pp. 23–25.

STANDARDIZATION, CERTIFICATION, QUALITY, AND SAFETY

ASPECTS OF PRODUCTION OF FUNCTIONAL EMULSION FOODS

L. V. Tereshchuk and K. V. Starovoitova

Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone:/fax: +7 (3842) 39-68-51, e-mail: terechuk_l@mail.ru

Received February 26, 2013; accepted in revised form May 28, 2013

Abstract: Criteria for evaluation of functional properties of emulsion foods are formulated. Balanced fat bases of emulsion sauces are simulated by using liquid vegetable oils of various fatty acid groups: oleic, linoleic, and linolenic. The optimum ratio of the components of an antioxidant-emulsifying complex is established. The efficiency of the antioxidant-emulsifying complex (AEC) for the emulsion sauce technology is experimentally confirmed with the help of sea-buckthorn or red palm oils and lecithin. It is established that the introduction of the AEC into the fatty base of emulsion products promotes the deceleration of oxidation processes in the product. The new emulsion sauce recipes and technology are scientifically justified.

Key words: emulsion sauce, antioxidant, lecithin, sea-buckthorn oil, red palm oil

UDC 664.3:615.451.451.23
DOI 10.12737/2058

INTRODUCTION

The concept of rational nutrition that underlies the current ideas of nutrition and health postulates the need for a new approach to the composition, properties, and, consequently, technologies of food products, which should not only meet the needs of the human organism in foodstuffs and energy but also provide it with the whole range of necessary macro- and microingredients, contributing to the prevention of alimentary-dependent diseases and preserving human health and longevity.

The new generation of food products includes those enriched with physiologically functional ingredients (functional products and enriched foodstuffs) designed to be consumed within food rations by all age groups of the healthy population and to reduce the risk of food-related diseases.

Physiologically, functional food ingredients can render a favorable effect on one or several physiological functions and metabolic processes in the human organism as they are consumed systematically in amounts of 10 to 50% of the daily physiological standard.

Fatty products for healthy nutrition must have a high nutritional value and contain the necessary set of polyunsaturated fatty acids (PUFAs), and the ratio of ω-3 to ω-6 acid families must be 1 : (5–10) at the optimal daily intake of specific acids. This ratio can be ensured by the necessary set and combination of vegetable oils, including blended oils.

Vegetable oils with high contents of PUFAs and balanced fatty acid compositions make it possible to produce emulsion fatty products of high biological efficiency. In addition, we should note that the oxidation of oils and fats in products with high contents of PUFAs and fat-soluble vitamins is the main factor that reduces their shelf life; therefore, the prevention of the oxidation of lipids is a major problem during the production and storage of fatty products.

The oxidation of oils and fats is a complex radical–chain process. The initial products of oxidation are structurally different peroxides and hydroperoxides, which are called the *primary products of oxidation.* Their transformations result in the formation of the *secondary products of oxidation:* alcohols, aldehydes, ketones, and acids with various lengths of their carbon chains, as well as their diverse derivatives, which, accumulating in oil, take part in the formation of the order and flavor of oxidized oil [4].

Overall, the mechanism of fat oxidation and antioxidant effects can be represented as follows:

free radical R, derived from a fatty acid or from its acyl under the effect of several factors, interacts with oxygen and forms a peroxide radical:

$$R\cdot + O_2 \rightarrow ROO\cdot,$$

which can interact with another unsaturated fatty acid or its acyl, forming a new free radical and a hydroperoxide:

$$ROO\cdot + RH \rightarrow ROOH + R\cdot.$$

In the initial period, which is called the *induction period*, the reaction flows slowly. At this time, the process can be accelerated in the presence of *pro-oxidants* or, reversely, decelerated due to the effect of antioxidants.

As hydroperoxides accumulate and disintegrate, forming new radicals, the process accelerates sharply:

$$2ROOH \rightarrow ROO\cdot + RO\cdot + H_2O.$$

The oxidation rate depends on the fat–acid composition of oils; the position of an unsaturated fatty acid in the triacylglycerol molecule; the amount, position, and geometrical configuration of double linkages; the pres-

ence of trace quantities of lipoxygenases in the oil–fat raw material; humidity; temperature; the presence of mixed-valence metals; and light [8].

In fats containing polyunsaturated fatty acids, oxidation occurs much faster than in saturated fats. As is known, the oxidation rate of a fatty acid grows proportionately to the number of double linkages in a molecule and to the amount of methylene groups between each pair of double linkages. Thus, the ratio of oxidation rates of oleic and linoleic acids ranges from 1 : 12 to 1 : 40, depending on product type, and arachidonic and linolenic acids are oxidized three and two times faster than linoleic acid. The structure of PUFAS affects the structure of secondary products of oxidation, in particular, the formation of volatile low-molecular aldehydes and ketones with low flavor thresholds, whose presence in a product gives it the odor and flavor of rancid oil [4].

Fats and oils differ significantly in their natural oxidative stability, which depends not so much on the composition and structure of fatty acids but on the presence of natural antioxidants, such as tocopherols, tocotrienols, carotenoids, and phospholipids, which inhibit the chain reactions of free-radical oxidation.

Food antioxidants are substances that decelerate the oxidation of, primarily, unsaturated fatty acids within lipids. Depending on their action mechanism, the substances that decelerate the process of fat oxidation are divided into the following three groups.

1. Antioxidants, chemical compounds that bind free lipid radicals, forming low-active radicals and thus interrupting the self-oxidation reaction. This mechanism is used by phenolic antioxidants, such as, tocopherol.

The introduction of antioxidant AH leads to the formation of new radicals A, which are much more stable that radicals R, slowing down the reaction and finally, under certain conditions, decelerating it sharply:

$$AH + R\cdot \rightarrow A\cdot + RH$$
$$AH + ROO\cdot \rightarrow ROOH + A\cdot$$
$$A + R\cdot \rightarrow AR.$$

2. Antioxidant synergists, substances that amplify the activity of antioxidants but that themselves do not have or have weak antioxidant properties. Efficient synergists are deoxidants, for example, ascorbic acid, which is used to protect oil–fat products from oxidation. Synergists SH_2 can reduce the A radicals without reacting with the ROO radicals,

$$SH_2 + 2A\cdot \rightarrow S + 2AH.$$

3. Complex formers, substances that are also antioxidant synergists, but the mechanism of their action is based on the formation of chelate complexes with metals. Citric and milk acids, as well as their salts, and lecithin are used for this purpose [1].

Some compounds, like lecithins and lactates, perform complex functions. Antioxidants help extend the shelf life of food products, protecting them from the rancidification of oils, fats, and fat components.

In practice, mixtures of antioxidants and complex formers are used to achieve synergetic effects at the same or even lower concentrations.

The use of antioxidants in fatty products for healthy nutrition must comply with the rational nutrition concept, preferably using natural food additives that are effective at low concentrations.

Taking into consideration the above, the design of oxidation-resistant emulsion fatty products for healthy nutrition is of scientific and practical interest, which requires broader basic and applied research and the incessant focus of the producers on this group of products.

GOAL AND OBJECTIVES OF RESEARCH

The goal of this paper is to develop recipes and to evaluate the quality of emulsion sauces balanced by their fatty-acid composition and containing an antioxidant-emulsifying complex, which includes natural carotenoids, tocopherols, and phospholipids.

The following objectives were set to meet the goal: to design a balanced fatty base of an emulsion salad sauce, including the ω-3 and ω-6 fatty acids; to study and analyze the composition and properties of lecithin and vegetable oils with high carotenoid and tocopherol contents; to investigate the synergetic effect of tocopherols, carotenoids, and phospholipids; to produce the antioxidant-emulsifying complex for emulsion sauces and to probe into its antioxidant properties; to develop the recipes and technology of oxidation-resistant emulsion sauces; and to examine the quality parameters of the developed sauces during their storage.

OBJECTS AND METHODS OF RESEARCH

Pursuant to the objectives set, we used generally accepted and original methods of research, including gas-liquid chromatography and photocolorimetry.

Crude fat was sampled and prepared in line with the requirements of ISO 5555-91 "Animal and vegetable fats and oils. Sampling" and ISO 661-89 "Animal and vegetable fats and oils. Preparation of test sample."

When studying the physicochemical parameters of vegetable oils and mayonnaise sauces, we determine:

- density with the help of a pyknometer, which represents a small glass vessel with a ground plug, whose neck has a mark to show the filling limit;
- melting temperature in a capillary vessel, open at both ends;
- acid number with the titration method. The method is based on dissolving oil in an ester–alcohol mixture (2 : 1) with the subsequent quick titration of the sample by an alkali in the presence of a phenolphthalein indicator until faint pink coloring;
- peroxide number by a method based on the interaction reaction of oil and fat oxidation products (peroxides and hydroperoxides) with potassium iodide in a solution of acetic acid and chloroform with the subsequent quantitative determination of the precipitated sodium thiosulfate;
- iodine number by the Hanus method. This method is based on the use of bromine iodide (IBr_2) as a reagent, which is formed by mixing bromine with iodine in glacial acetic acid. Bromine iodide associates with the double linkages of unsaturated fatty acids, and its surplus is titrated by sodium thiosulfate in the presence of potassium iodide and water;
- the quantity of β-carotene and tocopherols in oil was determined by the colorimetric method;
- the fatty-acid composition of oil was determined

by gas-liquid chromatography. The determination of the fatty-acid composition was preceded by converting fatty acids into methyl esters. The obtained chromatograms of the methyl esters of fatty acids were identified, and the quantitative content of fatty acids was calculated by peak areas in percent, using the standard methods; and

- the oxidation resistance of oils was determined by accelerated oxidation, when oils were kept at room temperature in standard conditions.

RESULTS AND DISCUSSION

The main aspects of the formation of functional properties of mayonnaise sauces imply solving the following problems:

the reduction of the product's caloric capacity by changing its ratio of fat phase to water phase. To decrease product fatness, part of oil was replaced with the water phase using natural emulsifying additives that ensure the required product texture;

the fatty-acid composition of the fat phase of emulsion sauces was improved to reach the recommended balance between saturated, monounsaturated, and polyunsaturated fatty acids by admixing (blending) oils in which various fatty acids prevailed;

increasing shelf life and preventing microbiological, hydrolytic, and oxidative spoilage by using natural highly active antioxidant additives, including tocopherols, carotenoids, and phospholipids.

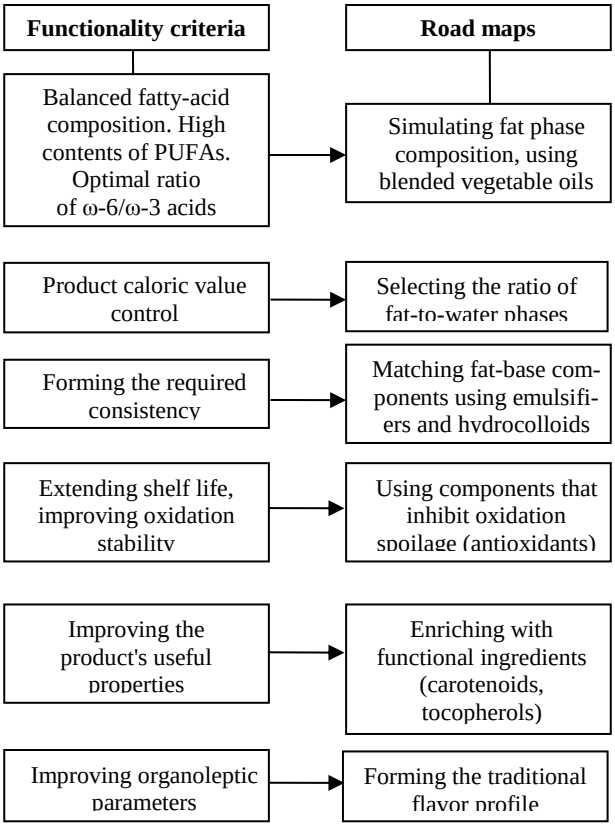


Fig. 1. Functionality criteria of emulsion products.

To justify the choice of vegetable oils used as fat-base components, we investigated the fatty-acid composition of several liquid vegetable oils.

The fatty-acid composition of vegetable oils, calculated from the chromatograms, is given in Table 1.

Table 1. Fatty-acid composition of vegetable oils

Fatty acids	Fatty acid contents, g/100 g of product			
	Refined vegetable oils			
	sunflower	sunflower high oleic	soybean	rape-seed
Total fatty acids	**94.90**	**97.90**	**94.90**	**95.40**
Saturated, including:	**11.30**	**10.60**	**13.90**	**6. 68**
oleic	0	0	0	0
caproic	0	0	0	0
caprylic	0	0	0	0
capric	0	0	0	0
lauric	0	0	0	0
myristic	0	0	traces	0
palmic	6.20	4.20	10.30	4.80
stearic	4.10	4.20	3.50	1.40
arachidic	0.30	0.60	traces	0.30
behenic	0.70	0.90	traces	0.20
Monounsaturated, including:	**23.80**	**69.0**	**19.80**	**56.30**
caproleic	0	0	0	0
lauroleic	0	0	0	0
myristoleic	0	0	0	0
palmitoleic	traces	1.70	0	0.30
oleinic	23.70	67.30	19.80	54.00
gadoleic	0	0	0	1.00
erucic	0	0	0	1.00
Polyunsaturated, including:	**59.80**	**18.30**	**61.20**	**32.40**
linoleic	59.80	18.30	50.90	22.50
linolenic	0	0	10.30	9.90
arachidonic	0	0	0	traces

We chose high oleic sunflower oil as a fat-base component. Sunflower high-oleic oil in its biochemical composition is very close to olive oil, which allows us to use it alongside olive oil in sauce recipes and to obtain a product with similar characteristics and properties. When designing balanced fat bases for mayonnaise sauces, we chose rapeseed oil alongside sunflower high-oleic oil. Note that the fatty-acid composition of low-erucic rapeseed oil is characterized by a very low level of saturated fatty acids, a relatively high level of monounsaturated fatty acids, and a mean level of polyunsaturated fatty acids. Rapeseed oil is a source of linolenic acid, which is absent in sunflower oil. We also considered the possibility of introducing soybean oil as a source of linoleic and linolenic acids into the fat base of mayonnaise sauces.

When choosing a composition of vegetable oils for the design of the fat base of functional mayonnaise sauces, we were guided by the following:

- reaching the $\omega_6 : \omega_3$ ratio of fatty acids in triacylglycerols close to the optimum, ensuring the therapeutic properties of products, namely, hypocholesterolemic and hypolipidemic effects;

- reaching in hypocholesterols a fat phase containing 2% of linolenic acid (of the total content of fatty acids), ensuring antisclerotic effects in combination with vitamins E and β-carotene; and

- ensuring the oxidation stability of the finished product.

The most rational way of creating a balanced fatty-acid composition of the fat base and controlling the ratio of the $\omega_3 : \omega_6$ essential fatty acids is the blending of vegetable oils that belong to different fatty-acid groups.

We studied the possibility of creating composite mixtures of vegetable oils with the required fatty-acid composition, controlled in line with the current requirements of the balanced nutrition concept. The biological efficiency of the calculated compositions was evaluated by the degree of approximation of their fatty-acid composition to the biologically optimal ratio of the $\omega_6 : \omega_3$ fatty acids, 5 : 1–10 : 1.

We propose that the recipes of emulsion sauces include two-component mixtures of vegetable oils consisting of sunflower high-oleic and rapeseed oils (70 : 30) and (60 : 40), as well as sunflower high-oleic and soybean oils (50 : 50). The fatty-acid composition of the chosen vegetable oils and the two-component mixtures are given in Table 2.

Table 2. Fatty-acid composition of vegetable oils in binary mixtures of vegetable oils

Fatty acids	Fatty-acid content, % of the total					
	sunflower oil (S_ho)	rapeseed oil (Ro)	soybean oil (So)	Two-component mixture		
				S_ho / Ro (70 : 30)	S_ho / So (50 : 50)	S_ho / Ro (60 : 40)
SFAs	10.60	6.68	3.90	9.40	7.30	8.60
MUFAs	69.00	56.30	19.80	65.20	44.40	62.60
PUFAs	18.30	32.40	61.20	22.50	39.70	20.40
Including:						
($C_{18:2}$) (ω_6)	18.30	22.50	50.90	19.60	34.60	20.40
($C_{18:3}$) (ω_3)	–	9.90	10.30	2.90	5.10	5.00
$\omega_6 : \omega_3$	–	2 : 1	5 : 1	7 : 1	7 : 1	5 : 1

* Legend: SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; ($C_{18:2}$), linoleic acid; ($C_{18:3}$), linolenic acid; S_ho, sunflower high-oleic oil; Ro, rapeseed oil; and So, soybean oil.

Thus, the designed compositions ensure ratios of linoleic acid (ω_6) to linolenic acid (ω_3) in a lipidic complex of (5.0 : 1.0) and (7.0 : 1.0), i.e., close to the optimal ratio of fatty acids that is responsible for the therapeutic properties.

The determinative factor in developing mayonnaise sauce technologies is the maximum preservation of the native state of the composition and properties of the finished products during storage. It is important to study the characteristics of the fat-phase oxidation process in mayonnaise emulsion sauces during storage and the regularities that are responsible for the rate and direction of this process.

The causes of spoilage of mayonnaise sauces are oxidation processes, resulting in the formation of decomposition products of fatty acids; the oxidation of triglycerides and the isomerization of initial acids. The accumulation of oxidation substances, such as peroxides, hydroperoxides, aldehydes, ketones, and oxy compounds, impairs the organoleptic and rheological properties of products, and reduces their physiological and biological value.

We suggest that the formulas of emulsion sauces should include an antioxidant-emulsifying complex, obtained by mixing natural phospholipids (lecithin) and vegetable oils, rich in natural antioxidants (carotenoids and tocopherols), and we suggest red palm oil and sea-buckthorn oil as such.

We have chosen these products because they contain antioxidantly and biologically effective substances, important for the human organism.

The formula of mayonnaise sauces should have an antioxidant composition: carotenoids–tocopherols–phospholipids.

We investigated the antioxidant effect of the tocopherols and carotenoids of red palm oil and sea-buckthorn oil blended with phospholipids on the oxidizing ability of oils during storage.

The quality of the samples under study was controlled by determining oxidative spoilage indicators, the peroxide number and the acid number. In order to intensify the process, we used accelerated oxidation: the samples were stored at room temperature, in the light, and with free access of air. The following oil compositions used to produce mayonnaise sauces served as check samples without adding antioxidant components:

- composition 1, a mixture of vegetable oils (check);
- composition 2, vegetable oils / phospholipids;
- composition 3, vegetable oils / red palm oil;
- composition 4, vegetable oils / sea-buckthorn oil;
- composition 5, vegetable oils / red palm oil / phospholipids; and
- composition 6, vegetable oils / sea-buckthorn oil / phospholipids.

The dynamics of the peroxide and acid numbers during the storage of fatty compositions with red palm and sea-buckthorn oils and their mixtures with phospholipids vs. the composition of vegetable oils without phospholipids and oils rich in carotenoids and tocopherols are shown in Figs. 3–5.

Analysis of the stated data shows that the peroxide number of the vegetable oil check sample without antioxidants increased from 1 to 17 mmol of active oxygen/kg. The peroxide number of the vegetable-oil compositions with phospholipids increased from 1 to 14.9 mmol of active oxygen/kg over 14 days of accelerated oxidation. The peroxide number of composition 3 increased from 1.9 to 12 mmol of active oxygen/kg over

14 days of accelerated oxidation. When introducing (red palm and sea-buckthorn) oils with antioxidant effects in combination with phospholipids, we observed the lowest growth of the peroxide number: it increased from 1 to 10.1 mmol of active oxygen/kg.

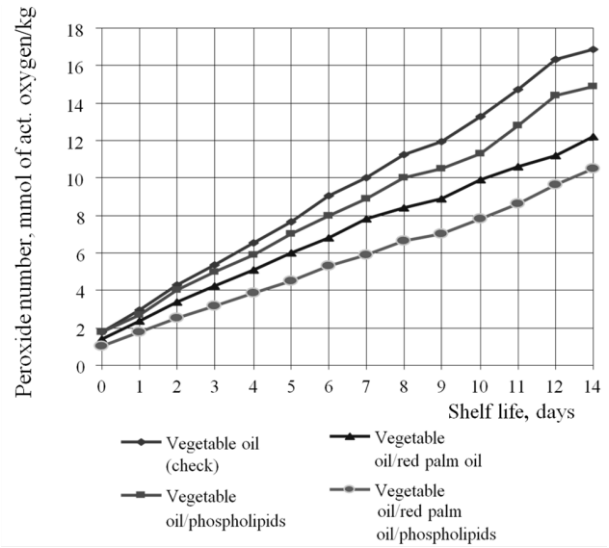


Fig. 2. Peroxide number dynamics during the oxidation of fatty compositions with red palm oil, red palm oil and phospholipids, and without adding such.

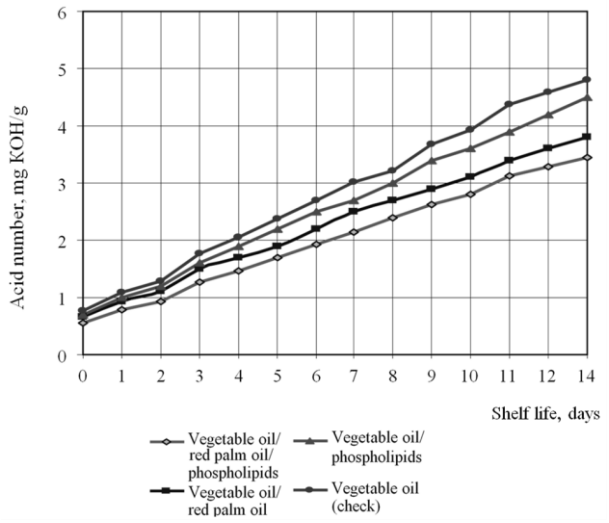


Fig. 3. Acid number dynamics during the oxidation of fatty compositions with red palm oil, red palm oil and phospholipids, and without adding such.

Comparison of the results allows us to conclude that red palm and sea-buckthorn oils in combination with phospholipids have antioxidant properties, can slow down oxidation processes in unsaturated fatty acids, and display a synergetic effect during their joint introduction into fatty compositions.

It follows from the above that natural carotenoids, tocopherols, and phospholipids can be used as components of fatty emulsion products to improve the antioxidant potential of fat phases.

It has been established that the introduction of emulsion products of the antioxidant-emulsifying complex based on red palm and sea-buckthorn oils into the fat base helps increase the shelf life of finished products.

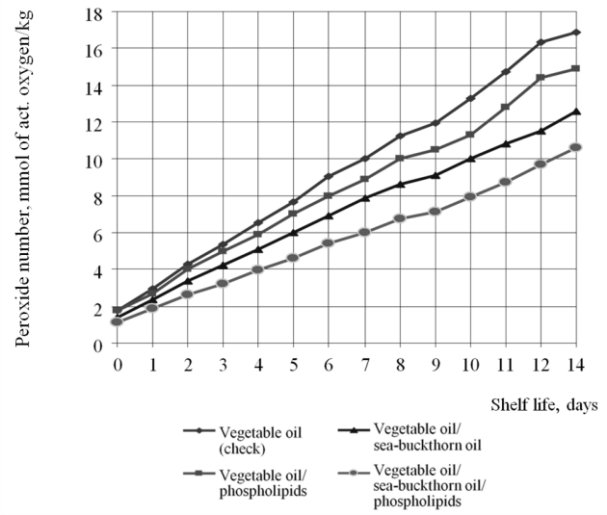


Fig. 4. Peroxide number dynamics during the oxidation of fatty compositions with sea-buckthorn oil, sea-buckthorn oil and phospholipids, and without adding them.

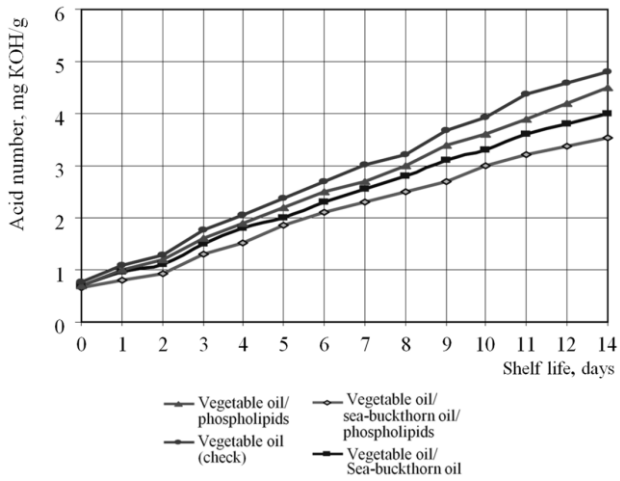


Fig. 5. Acid number dynamics during the oxidation of fatty compositions with sea-buckthorn oil, sea-buckthorn oil and phospholipids, and without adding them.

The antioxidant-emulsifying complex is developed by mixing food phospholipids with vegetable oils. For their uniform distribution in the fat base and increased efficiency, phospholipids and vegetable (sea-buckthorn or red palm) oils are dissolved in deodorized oil at 60–70^{0}C in ratios of 1 : 4–1 : 5, which corresponds to the following percentage: refined deodorized vegetable oil, 80%; phospholipids, 10 %; and red palm oil, 10 %.

For sea-buckthorn oil, the ratio is the following: refined deodorized vegetable oil, 85 %; phospholipids, 10 %; and sea-buckthorn oil, 5%.

The new product is characterized by high contents of carotenoids, 65–70 mg/100 g, and tocopherols, 45–50 mg/100 g (Table 3).

Table 3. Tocopherol and carotenoid contents in the antioxidant-emulsifying complex (AEC)

Parameter	AEC based on	
	red palm oil (AEC 1)	sea-buckthorn oil (AEC 2)
Vitamin E, mg/100 g	43	46
Carotenoids, mg/100 g	67	69
including:		
β-carotene	21	23

The physicochemical and organoleptic parameters of the AEC based on red palm and sea-buckthorn oils with phospholipids are given in Table 4.

Table 4. Physicochemical and organoleptic parameters of the antioxidant-emulsifying complex

Parameter	AEC with	
	red palm oil (AEC 1)	sea-buckthorn oil (AEC 2)
Consistency	Fluid	
Color	Orange-yellow	
Odor and flavor	Low, typical of vegetable oil used and phospholipids. Fusty, sour, or any foreign odors are inadmissible.	
Mass fraction of moisture and volatile substances, %	0.3 ± 0.05	0.3 ± 0.05
Mass fraction of phospholipids, %	10.0 ± 0.05	10.0 ± 0.05
Mass fraction of vegetable oil, %	80.0 ± 0.05	85.0 ± 0.05
Mass fraction of red palm/sea-buckthorn oil, %	10 ± 0.05	5 ± 0.05
Acid number of oil, mg KOH/g, no more than	2.5	3.5
Peroxide number, mmol of active oxygen/kg, no more than	10.0	10.0
Density (15°C) g/cm^3	0.924	0.922
Refractive index (20°C)	1.474	1.476
Viscosity (20°C), c П	55.1	58.4
Iodine number, % J_2	123	126
Vitamin E, mg/100 g	43	46
Carotenoids, mg/100 g	67	69
including:		
β-carotene	21	23

Egg products are traditionally used to produce mayonnaises as emulsifiers; the main emulsifier in mayonnaise is egg yolk, more precisely, lecithin and other phospholipids contained in it. In addition to its emulsifying properties, yolk affects the organoleptic characteristics of products (flavor and consistency).

Unlike mayonnaises, salad sauces do not contain egg products. The need arises to select the right emulsifier that would replace egg powder in terms of nutritive value and that would allow us to produce stable emulsions with the required viscosity, rheology, and possibly longer shelf life.

We have developed a antioxidant-emulsifying complex that contains 10% of phospholipids.

Note that food vegetable phospholipids are used successfully as emulsifiers to produce dietary emulsion products, such as margarines, mayonnaises, and spreads. In addition, lecithins are used as liquefiers of chocolate mass and as stabilizers of various jelly products.

The main functions of phospholipids in food products are related to emulsification, viz., the ability to form and keep in a homogeneous state both oil-in-water and water-in-oil emulsions, as well as to stabilize various systems. The above functions make phospholipids traditional food additives, deliberately put into food products to give them the required properties, to improve their antioxidant potential, and to increase their shelf life, since some phospholipids display antioxidant effects.

However, the use of phospholipids in the emulsion product technology is not limited to solving only technological problems; their high physiological activity preconditions the creation of new biologically wholesome fatty products.

Our research has shown that food vegetable phospholipids have high biological activity, which manifests itself in favorable effects on lipid exchange and the liver functionality, reducing hypercholesterolemia and improving the antioxidative functions of the human organism. As natural emulsifiers, phospholipids ensure the transfer of fat-soluble vitamins, promoting their oxidation in the liver, conversion in the tissues, etc.

Proceeding from the biological activity of phospholipids and the current ideas of balanced nutrition, it has been established that the average food ration of an adult must contain 3.5–5.0 g of lecithin [5].

Tables 5 and 6 show comparative evaluation of the physicochemical parameters and chemical composition of the antioxidant-emulsifying complex and egg powder.

Table 5. Comparative evaluation of the physicochemical parameters of food additives

Parameter	Parameter value		
	Egg powder	AEC with	
		red palm oil (AEC 1)	sea-buckthorn oil (AEC 2)
Mass fraction, *%:* Moisture and volatile substances	6.15–6.50	0.30–0.35	0.30–0.35
Lipids	33.10–34.15	78–86	78–86
Phospholipids	9.15–9.98	10	10
Proteins	45.20–45.90	absent	absent
Minerals	4.35–4.48	4.58–4.87	4.58–4.87
Cholesterol	2.30–2.40	absent	absent
Acid number of lipids isolated from the product, mg KOH/g	4.50–4.75	0.90–1.10	1.0–1.20
Peroxide number of lipids isolated from the product, mol of active oxygen /kg	5.15–5.30	2.28–2.58	2.48–2.88

Comparative analysis of the physicochemical parameters and chemical composition of the examined additives for the production of emulsion sauces has shown that the AEC qualitative composition is an alter-

native to egg powder.

Thus, the selection of surface-active substances is of special importance for the technology of low-fat combined emulsions, including salad sauces. A pertinent system of emulsifiers helps obtain highly stable products of various compositions and reduced caloricity. In addition, the main concepts of choosing emulsifying–stabilizing systems should primarily be focused on the group of natural compounds and their synthetic analogs with the maximum functionality that help create a broad range of emulsion products with preset properties.

The complex studies undertaken have helped us develop recipes for low-calorie emulsion sauces that do not contain animal components.

Table 6. Chemical composition and nutritive value of food additives

Parameter	Parameter value	
Mass fraction of vitamins, mg/100 g:	Egg powder	AEC
E	absent	43–46
β-carotene	0.15	22
Mass fraction of polyunsaturated fatty acids, %	10.15–10.60	43.20–43.60

Table 7. Recipes of functional emulsion sauces

Components	Content of recipe components, %	
	Sauce of 35% fatness	Sauce of 45% fatness
Two-component mixture of vegetable oils	25.0	35.0
AEC	10	10
Total fats	35.0	45.0
Stabilizer	3.8	2.9
Sugar sand	2.0	2.0
Food cooking salt Extra	1.0	1.0
Mustard	0.75	0.75
Milk acid of 80%	0.34	0.34
Sodium benzoate	0.2	0.2
Flavoring agent Yolk	0.008	0.008
Water	56.9	47.8
Total	100	100

* Legend: AEC 1, the antioxidant-emulsifying complex with red palm oil; AEC 2, the antioxidant-emulsifying complex with sea-buckthorn oil.

When designing the recipes of emulsion sauces, we took into account consumer preferences in sauce caloricity. We have proposed emulsion sauce recipes with fat mass fractions of 35 and 45%.

The recipes of functional emulsion sauces are given in Table 7.

A special feature of the proposed technology is the exclusion of the repasteurization stage due to the absence of egg products in the mayonnaise sauce recipe. The classical scheme of mayonnaise production envisages that, before the introduction of egg products, the mayonnaise paste, pasteurized at 80°C, is cooled down to 60°C to avoid the denaturation of the albumen of the egg products. After the introduction of egg products, the mixture is repasteurized. We propose that the AEC, which replaces egg powder, is to be introduced in the amount of 10% at the emulsification stage at 35–40°C, which allows preserving the vitamin complex.

Longer shelf life is a priority in creating new products and technologies.

Emulsion sauces, due to their specific composition, are products unstable during storage; therefore, the longer shelf life of these products is very topical. The use of the AEC as a component containing natural antioxidants, such as tocopherols, carotenoids, and phospholipids, slows down oxidation processes during storage.

The studied samples of emulsion salad sauces were kept in consumer packaging, made of polymer materials and admitted for use by the Russian Ministry of Public Health.

The emulsion sauces were stored at 4 ± 2°C for 9 months.

The intensity of oxidation and hydrolysis processes during storage was studied, and the dynamics of the peroxide number of the fat phase of emulsion sauces was analyzed. The dynamics of the peroxide number of the developed emulsion sauces during storage is given in Fig. 6.

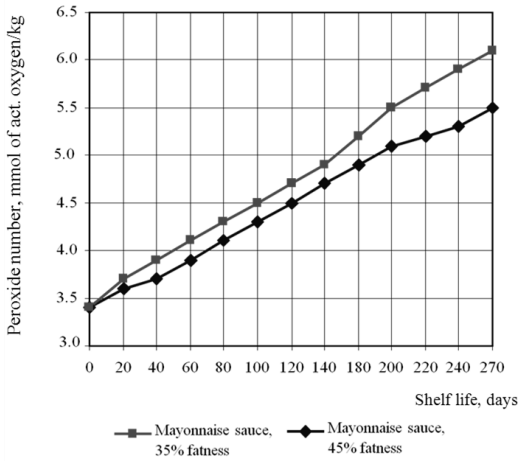


Fig. 6. Peroxide number dynamics during the storage of emulsion sauces at 4 ± 2°C.

Analyzing the storage dynamics of peroxide numbers, we may conclude that the intensity of accumulation of primary oxidation products increases as the mass fraction of fat in emulsion sauces decreases, which is associated with the intensification of the hydrolysis process. Thus, the peroxide number of a sauce containing 35% of the fat phase increased from 3.4 to 6.2 mmol of active oxygen/kg over 9 months of storage at 4 ± 2°C, by stayed within the set standard (no more than 10 mmol of active oxygen/kg).

Changes in the organoleptic parameters of the quality of emulsion sauces during storage at 4 ± 2°C are given in Table 8.

Table 8. Organoleptic parameters of the quality of emulsion sauces during storage

Month number	Appearance, consistency	Odor and flavor	Color
	Standard requirements		
	Uniform creamy product; singular air bubbles are admissible.	Flavor is slightly pungent, sourish, with the odor and flavor of the introduced flavoring and aromatic additives	From light yellow to yellow, uniform throughout its mass.
	Emulsion sauce		
1	Uniform creamy product with singular air bubbles	Pleasant, sourish, without marked signs of bitterness	From light yellow to yellow, uniform throughout its mass.
2			
6			
4			
5			
6			
7			
8			
9			

It follows from the data in Table 8 that all parameters remain within the standard during storage.Changes in the physicochemical parameters of the quality of emulsion sauces during storage are given in Table 9.

Table 9. Physicochemical parameters of the quality of emulsion sauces during storage

Parameters	Parameters of emulsion sauces (after 9 months of storage), with fatness of		Standard requirement
	45%	35%	
Mass fraction of fat, %	45.0 ± 0.5	35.0 ± 0.5	At least 15
Mass fraction of moisture, %,	62.0 ± 0.5	52.0 ± 0.5	In line with product specifications
Acidity, % of conversion to acetic acid	0.7 ± 0.1	0.8 ± 0.1	No more than 1.0
Emulsion stability, % of intact emulsion	99.0 ± 0.5	99.0 ± 0.5	At least 98
Hydrogen index (pH) at 20^0C	4.5 ± 0.2	4.2 ± 0.2	4.0–4.7
Effective viscosity at 20^0C and a shear rate of $3s^{-1}$, Pa/s	15 ± 0.0	13 ± 0.0	5.0–20.0

Thus, the table data show that the physicochemical parameters of the sauces under study did not change and remained within the standard during storage. Data about the microbiological parameters of emulsion sauces during storage are given in Table 10.

Table 10. Microbiological parameters of the quality of emulsion sauces during storage

Parameters	Shelf life of emulsion sauces at 4 ± 2°C, days										Standard
	Emulsion sauce of 45%					Emulsion sauce of 35%					
	0	60	120	180	270	0	60	120	180	270	
(Coliform bacteria), absent in g	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.1
Pathogens, including salmonellae absent in g	25	25	25	25	25	25	25	25	25	25	absent in 25 g
staphylococci, absent in g	Not found	Not found	Not found	Not found	Not found	Not found	Not found	Not found	Not found	Not found	Inadmissible
QMAFAnM, CFU/g	Not found	Not found.	Not found.	Not found.	Not found.	Not found.	Not found.	Not found.	Not found.	Not found.	Inadmissible
Yeast, CFU/g	50	55	70	110	160	55	80	90	130	180	$5*10^2$
Mold, CFU/g	0	2	5	8	15	0	3	8	14	20	50

Table 11. Nutritive and energy values of emulsion sauces

Parameter	Emulsion sauce of 35% fatness	Emulsion sauce of 45% fatness
Proteins,%	1.62 ± 0.01	1.62 ± 0.01
Lipids, %	35.0 ± 0.1	45.0 ± 0.1
including phospholipids	1.0 ± 0.1	1.0 ± 0.1
Linoleic acid, %	12.1 ± 0.1	15.5 ± 0.1
Linolenic acid, %	1.73 ± 0.01	2.2 ± 0.1
Carbohydrates, %	3.9 ± 0.1	3.9 ± 0.1
Carotenoids, mg/100 g	6.7 ± 0.1	6.7 ± 0.1
Tocopherols, mg/100 g	4.3 ± 0.1	4.3 ± 0.1
Energy value, kcal	326	427

Analyzing the obtained data we may conclude that the samples of emulsion sauces of different fatness are characterized by high microbiological purity both on the day of production and during the whole shelf life Coliform bacteria were absent in 0.01 g of products at the end of the shelf life. *Staphyloccocus aureus* and pathogenic microorganisms, including salmonellae, were not found in the standardized masses of products through the whole storage period. Yeast and mold were also within the standard. Thus, during the whole storage period the growth of microorganisms was insignificant, and the sauces preserved sufficient microbiological purity. The positive results of essaying the safety of emulsion sauces allowed us to establish the shelf life of 7 months at a temperature regime of 4 ± 2^oC.

Data about the nutritive and energy values of the developed emulsion sauces are given in Table 11.

The above data show that the developed mayonnaise sauces have low caloricity and a balanced ratio of ω_6 : ω_3 fatty acids; contain physiologically valuable ingredients, such as phospholipids, carotenoids, and tocopherols, in amounts that comply with the standard physiological needs for food substances; and can be used as functional food products.

REFERENCES

1. Doronin, A.F., Ipatova, L.V., Kochetkova A.A., Nechaev, A.P., Khurshudyan, S.A., and Shubina, O.G., *Funktsional'nye pishchevye produkty* (Functional Food Products), ed. by A. A. Kochetkova, Moscow: DeLi print, 2008.
2. Ipatova, L.V., Kochetkova, A.A., Nechaev, A.P., and Tutel'yan V.A., *Zhirovye produkty dlya zdorovogo pitaniya: sovremennyi vzglyad* (Fatty Products for Healthy Nutrition: A Contemporary View), Moscow: DeLi print, 2009.
3. Korneva, E.P., Kalmanovich, S.A., Martovshchuk, E.V., et al., *Ekspertiza masel, zhirov i produktov ikh pererabotki. Kachestvo i bezopasnost'* (Examination of Oils, Fats, and Their Derivatives: Quality and Safety), ed. by V. M. Poznyakovskii, Novosibirsk: Izd. Sib. Univ., 2007.
4. *Understanding and Measuring the Shelf-Life of Food*, ed. by R. Steele, New York: Woodhead Publishing, 2004; St. Petersburg: Professiya, 2006.
5. Tutel'yan, V.A., Nechaev, A.P., and Kochetkova, A.A., Funktsional'nye zhirovye produkty v structure pitaniya (Functional fatty products in the nutrition structure), *Maslozhirovaya promyshlennost; (Oil and fat industry)*, 2009, no. 6, pp. 6–9.
6. Tereshchuk, L.V. and Umanskii M.S., *Molochno-zhirovye kompozitsii: aspekty konstruirovaniya i ispol'zovaniya: mohografiya* (Milk Fat Compositions: Aspects of Design and Usage: A Monograph), Kemerovo: Kemerovskii Tekhnologicheskii Institut Pishchevoi Promyshnlennosti, 2006.
7. Valdes, A.F. and Garcia, A.B., A Study of the evolution of the physicochemical and structural characteristics of olive and sunflower oils after heating at frying temperatures, *Food chemistry*, 2006, vol. 98, no. 2, pp. 214–219.
8. Kühn, M.C., European Functional Foods: Challenges beyond the Regulatory Milestone, *Food Highlights*, 2007, no. 15, pp. 1–11.
9. *Handbook of Nutriceuticals and Functional Foods*, Ed. by R. E. S. Wildman, New York: CRC Press, 2007.
10. Rubalya, V.S. and Neelamegam, P., Antioxidant potential in vegetable oil, *Research Journal of Chemistry and Environment*, 2012, vol. 16, no. 2, pp. 87–94.

A STUDY OF THE COMPLEXING AND GELLING ABILITIES OF PECTIC SUBSTANCES

O. V. Salishcheva and D. V. Donya

Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone:/fax: +7 (3842) 39-68-64, e-mail: platchim@rambler.ru

Received April 1, 2013; accepted in revised form May 31, 2013

Abstract: Cranberries, lingonberries, blueberries, and food systems based on these berries with different solvents and sugars have been studied. A physicochemical analysis of cranberries, lingonberries, and blueberries has been conducted. Crystalline pectin from cranberries, lingonberries, and blueberries has been isolated to determine the degree of etherification. The gelling ability of the pectic substances has been studied. The effect of different solvents and sugars on the rheological properties of food systems containing pectin has been examined. A comparative estimation of the gelling ability of the pectic substances contained in cranberries, lingonberries, and blueberries and in chitosan and alginate gelling agents has been conducted. The viscous properties have been found to increase in the series: cranberry pectin < lingonberry pectin < blueberry pectin < sodium alginate < chitosan. The complexing ability of pectins with respect to copper and iron ions has been studied and compared to that of casein. Casein exhibits a lower complexing ability with respect to iron ions than pectin. It has been found that the complexing properties of pectin vary with its concentration: the more dilute the solution, the higher the complexing ability of pectin.

Key words: pectic substances, gelling ability, blueberries, cranberries, lingonberries, rheological properties, complexing ability

UDC 664.292
DOI 10.12737/2172

INTRODUCTION

The food industry is being rapidly developed. At the same time, the competition between manufacturers is escalating. The manufacturers strive to anticipate the customer needs by paying ever-increasing attention to the quality of products and introducing advanced technologies. The perception and attitude of consumers to food products have a huge impact on the sales of the products. These requirements have led to the formulation of a new generation of foodstuffs that favorably affect the human body [1, 2].

The most important curative measure aimed at reducing the adverse effects of physical, chemical, and biological factors of the environment on human health is the use of pectin preparations approved by the Ministry of Health of the Russian Federation in the healthful and dietary meals of all groups of the population.

Pectins are widely used in all sectors of the food industry. Pectin is a purified hydrocarbon obtained by extraction from plant raw materials. Pectins are used as gelling, stabilizing, thickening, water-retaining, and clarifying agents, as substances facilitating filtering, and as a means for encapsulation. For example, in the dairy industry, pectins are actively used in the manufacture of yogurts, cheese, ice cream, milk–fruit desserts, and fermented and acidified dairy products; pectins are used as an emulsifier for the manufacture of mayonnaise and liquid margarines in the fat-and-oil industry. Pectins are successfully used for the production of marmalade, jelly fillings, whipped confectionery products, such as marshmallows and pastille, and candy pastes.

In the European system of codification of food additives, pectin has the number E440.

According to the current nomenclature, pectic substances include protopectin, pectin, pectinic acid and pectinates, pectic acid and pectates [3].

- Protopectin is water-insoluble natural pectin with a complex structure, which has not yet been precisely specified. It is believed that its composition includes all the above complexes.
- Pectin, or soluble pectin—water-soluble polygalacturonic acids methoxylated to varying degrees—is formed from protopectin under the action of acids, alkalis, or protopectinase enzyme.
- Pectinic acid is macromolecular polygalacturonic acid with carboxyl groups partially etherified with methanol. Salts of pectinic acid are referred to as pectinates.
- Pectic acid results from complete demethoxylation of pectinic acid. The solubility of pectic acid is lower than that of pectinic acid. Salts of pectic acid are referred to as pectates.

The main functional feature of pectin as a gelling agent is the ability to form gels in aqueous solutions in the presence of a certain amount of sugar and acid or calcium ions. In addition, pectin can absorb and rid the body of biogenic toxins, anabolic steroids, xenobiotics, metabolites, and biologically harmful substances capable of accumulating in the body [4–6].

Thus, we should put emphasis on two main properties of pectin: complexing and gelling abilities, which are used depending on the sphere of application.

Any plant raw material with a high content of pectin can be used for the production of pectic substances. Four basic types of feedstock are processed: apple pomace, sugar beet pulp, sunflower heads, and citrus peels. These types of raw materials are not basic for the regions of Siberia and the Far East; therefore, the problem of the complete use of local fruit and berry resources is of particular importance. The present-day technologies for isolating pectin from plant raw materials involve the use of significant amounts of acids and ethanol. Data on the enzymatic hydrolysis of pectin-containing feedstocks are hardly available. The application of ultrafiltration and sorption on ion-exchangers in the technology for purification of extracts of beet pectin and some other types of pectins has been reported [3, 7].

Recently, studies on the isolation, purification, and examination of the properties of pectins derived from nonconventional plant raw materials have been developed. It is known that valuable sources of pectic substances are fruits and berries, including cranberries, lingonberries, and blueberries. These berries are abundant in the Siberian region because of their easy maintenance, high frost resistance, early ripening, special gustatory qualities, and a wide range of medicinal properties.

The chemical composition, properties, and total content of pectic substances, the ratio between protopectin and soluble pectin, and their acetyl component in the above feedstocks suggest that pectin of cranberries, lingonberries, and blueberries can be regarded as a promising structure-forming agent.

The gelling ability depends on the molecular weight of pectin, the degree of etherification of its molecule, the content of functional groups, the sugar concentration in the solution, the amount of ballast substances accompanying this pectin, the ambient temperature, and pH of the medium [3].

In selecting technological conditions for the manufacture of food products with a given structure, it is important to maximally preserve the gelling properties of pectic substances during the entire process, including packaging, transportation, and storage of the product. Therefore, it is necessary to study the impact of various factors on the gelling ability of pectic substances derived from various plant materials.

The aim of this study was to examine the gelling ability of pectic substances of cranberries, lingonberries, and blueberries and determine the complexing ability of pectins with respect to copper(II) and iron(III) ions.

In accordance with the stated objective, the following problems were solved:

- Physicochemical analysis of the original feedstock;
- Isolation of pectic substances from cranberries, lingonberries, and blueberries growing in the Kemerovo and Tomsk regions;
- Examination of the complexing ability of pectic substances of cranberries, lingonberries, and blueberries;
- Analysis of the effect of various solvents and sugars on the rheological properties of food systems containing pectic substances;
- Comparison of the gelling ability of pectic substances contained in cranberries, lingonberries, and blueberries and various gelling agents;
- Examination of the complexing ability of pectic substances with respect to copper(II) and iron(III) ions and comparison of the complexing ability of pectic substances and casein.

MATERIALS AND METHODS

Samples of frozen lingonberries, blueberries, and cranberries growing in the Kemerovo and Tomsk regions and powdered pectin (citrus pectin SS 200, Denmark) were studied.

The physicochemical analysis of the berries was as follows: (1) determination of the content of dry matter and moisture (GOST (State Standard) 28561-90); (2) identification of soluble and insoluble solids (GOST (State Standard) 29031-90); (3) determination of the total acidity (GOST (State Standard) 255555.0-82); (4) identification of nitrates (GOST (State Standard) 29270-95); (5) identification of pectic substances in the form of water-soluble pectins and protopectin (GOST (State Standard) 29059-91); and (6) determination of the degree of etherification of pectins (GOST (State Standard) 29059-91).

Absorbance was measured using a KFK-3 photoelectric colorimeter at a layer thickness of 1 cm.

Rheological experimental data were recorded using a Reotest-2 rotary viscometer.

EXAMINATION OF THE COMPLEXING ABILITY OF PECTIC SUBSTANCES

Heavy metals entering the body can cause a number of metabolic disorders, mostly in redox processes. The formation of metal bicomplexes with various cell components can lead to membrane damage and inhibition of the activity of various enzymes.

Copper is one of the heavy metals that can contaminate food products. The prevention of possible consequences of the penetration of copper into the human body is based on the binding of copper in the complexation with pectin.

In recent years, considerable attention has been paid to the determination of the structure of pectic substances owing to their valuable technical properties and high physiological activity. They have a wide biological action spectrum: many pectins have immunomodulatory effects and can rid the body of heavy metals, biogenic toxins, anabolic steroids, xenobiotics, metabolites, and biologically harmful substances capable of accumulating in the body: cholesterol, lipids, bile acids, and urea [8, 9].

A promising field of application of pectins can be the use of their modified oligomers as a template for the manufacture of extended-release drugs. There are data on studies of the antitumor activity of pectic substances that are products of fermentolysis of deetherified pumpkin pectin; their protective effect is significantly higher than the activity of homogalacturonic oligosaccharides derived from citrus pectin [3].

A variety of new physicochemical, complexing, and physiological properties can be imparted to pectins through chemical modification: etherification, amidation, and acylation [10, 11].

The complexing ability of pectin is based on its ability to form insoluble complex compounds with heavy metals and radionuclides (Fig. 1). It is this property that defines pectin as a prophylactic agent in contaminated and polluted areas, as recommended by the World Health Organization (WHO). The prophylactic daily dose of pectin is 4–5 g; under conditions of radioactive contamination, the daily dose is 15–16 g.

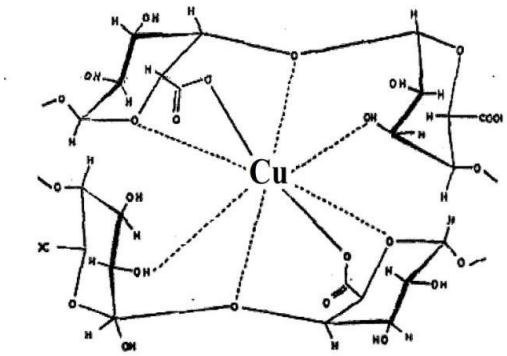


Fig. 1. Formation of a complex compound of pectin with copper ions.

The complexing properties of pectic substances depend on the content of free carboxyl groups, i.e., the degree of etherification of the carboxyl groups with methanol. The degree of etherification determines the linear charge density of the macromolecule, and consequently, the strength and mode of binding of the cations [12, 13].

At a high degree of etherification of pectin (above 90%), the free carboxyl groups that include C6 atoms are significantly spaced apart. In addition, the salts of pectic acid are almost completely dissociated. With a decrease in the degree of etherification, i.e., with an increase in the charge of the macromolecule, the binding between the pectic substances and the cations enhances and the stability constant of the pectates increases. At a degree of etherification of 40%, the conformation undergoes a change, which leads to the aggregation of pectin molecules and the formation of a strong intramolecular chelate bond [3].

The determination of the complexing ability of the analyte with respect to copper is based on the spectrophotometric detection of copper in the form of a copper ammine complex, which has an intense blue color with maximum absorption at 620 nm and is formed after the addition of excess ammonia to a solution comprising copper sulfate according to the reaction

$$CuSO_4 + 4NH_4OH \rightarrow [Cu(NH_3)_4]SO_4 + 4H_2O.$$

To specify the maximum absorption, a spectrophotometric absorption curve was plotted.

The concentration of copper in an aqueous pectin solution containing Cu^{2+} ions was determined form the absorbance of the solution.

To this end, the absorbance of a set of Cu^{2+} solutions with varying concentration was found. According to the derived experimental data, a calibration graph of absorbance versus Cu^{2+} ion concentration was plotted to find the concentration of copper ions in the pectin-containing solutions. The complexing ability value was found as the difference in the copper concentration in the aqueous solution and the pectin-containing solution. The results of the study are shown in Table 1.

Table 1. Results of examination of the complexing ability of pectin with respect to Cu^{2+} ions

m (pectin), mg	m_{add} (Cu^{2+}), mg	m_{free} (Cu^{2+}), mg	m_{bound} (Cu^{2+}), mg	Complexing ability mgCu^{2+}/g of pectin
5	400	1.2	398.8	79760
10	400	1.2	398.8	39880
15	400	1.2	398.8	26590

A comparative estimation of the complexing ability of pectin and casein has been conducted.

Casein belongs to a group of proteins referred to as phosphoproteins; it comprises a large number of phosphate groups that bind calcium.

Heavy metal ions are bound by casein through a phosphorus caseinate–calcium phosphate complex; this leads to the formation of water-insoluble salts.

To compare the binding ability of pectin with protein, a casein solution was prepared. A weighed portion of casein was dissolved in water with a sodium acetate additive under heating on a water bath. The analysis results are shown in Table 2.

Table 2. Results of examination of the complexing ability of casein with respect to Cu^{2+} ions

m (casein), mg	m_{add} (Cu^{2+}), mg	m_{free} (Cu^{2+}), mg	m_{bound} (Cu^{2+}), mg	Complexing ability mgCu^{2+}/g of casein
5	400	1.9	398.1	79620
10	400	2.1	397.9	39790
15	400	2.4	397.6	26500

To determine the complexing ability of pectin and casein with respect to iron ions, the absorbance of solutions containing $FeCl_3$ at λ = 395 nm was found. The analysis results are shown in Tables 3 and 4.

Table 3. Results of examination of the complexing ability of pectin with respect to Fe^{3+} ions

m (pectin), mg	m_{add} (Fe^{3+}), mg	m_{free} (Fe^{3+}), mg	m_{bound} (Fe^{3+}), mg	Complexing ability mgFe^{3+}/g of pectin
5	18.0	5.0	13.0	2600
10	18.0	9.0	9.0	900
15	18.0	12.4	5.6	370

Table 4. Results of examination of the complexing ability of casein with respect to Fe^{3+} ions

m (casein), mg	m_{add} (Fe^{3+}), mg	m_{free} (Fe^{3+}), mg	m_{bound} (Fe^{3+}), mg	Complexing ability mgFe^{3+}/g of casein
5	18.0	16.0	2.0	400
10	18.0	16.7	1.3	130
15	18.0	16.7	1.3	90

EXAMINATION OF THE GELLING ABILITY OF PECTIC SUBSTANCES

Gelation begins as follows. A hydration shell is formed around the pectin molecules in the solution and prevents the molecules from coming into contact with each other. Since the carboxyl groups of polygalacturonic acids undergo dissociation in the solution, the pectin molecules acquire a charge and mutually repel. The formation of a gel skeleton primarily requires the weakening or elimination of electrostatic repulsion forces. Since the acid contained in the solution is dissociated to a higher degree than polygalacturonic acid, the degree of dissociation of pectin is reduced; that is, the electrostatic charge of its particles decreases.

At the same time, under the effect of sugar, the pectin molecules undergo dehydration resulting in the appearance of some "bare" regions with no polarity. The formation of a structural skeleton occurs via the adhesion of individual molecules through their dehydrated regions under the action of intermolecular forces. The skeleton is strengthened owing to hydrogen bonds between the carboxyl and hydroxyl groups of the adjacent chains of the pectin molecules [3]. This interaction between the pectin molecules results in the formation of a cellular structure (Fig. 2).

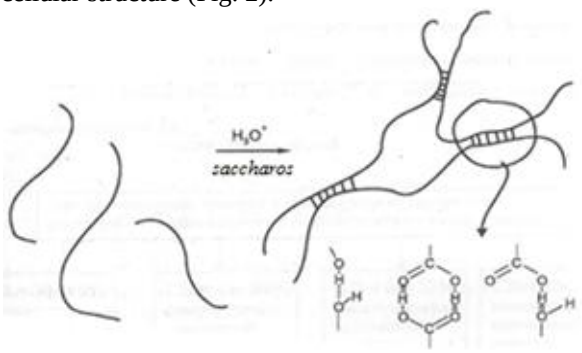


Fig. 2. Mechanism of gelation of high-etherified pectin [3].

The gelling ability of pectin depends on its molecular weight (degree of polymerization), because an increase in this quantity leads to an increase in the gel strength, on the amount of methyl groups contained in the pectin molecule (degree of methoxylation), the content of free carboxyl groups, and their binding by metals [3, 14, 15].

Depending on the degree of etherification of carboxyl groups, pectins are divided into high- and low-etherified (the degree of etherification is less than 50%), which are obtained from an original feedstock by acidic or alkaline extraction or by enzymatic hydrolysis. Pectins of different nature exhibit significantly different gelling ability. Pectins with a higher quality (high-etherified) are prepared from citrus and apple peels; pectins with a lower quality (low-etherified) are produced from sugar beet pulp.

At the first stage, the berries were subjected to a physicochemical analysis in order to determine (1) the content of solids and moisture, (2) the amount of soluble and insoluble solids, (3) the total acidity, (4) the nitrate content, (5) the amount of pectic substances in the form of water-soluble pectins and protopectin, and (6) the degree of etherification of pectins.

Crystallized pectin was isolated from the above berries by acid–ethanol extraction.

The quantitative analysis of pectic substances showed that the weight fraction of water-soluble pectin was 0.78% in blueberries, 0.77% in lingonberries, and 0.465% in cranberries, while the amount of water-insoluble pectin (protopectin) was 0.52, 0.13, and 0.255%, respectively. The total amount of pectin was 1.3% in blueberries, 0.9% in lingonberries, and 0.69% in cranberries. The resulting pectins had the following degree of etherification: 86.2, 63.4, and 75.9% in blueberries, lingonberries, and cranberries, respectively.

One of the important problems of the technology of food products is to give them desired form and structure. The structure type and mechanical properties of food products determine their texture. The texture is used to estimate the quality of the food product, which can be determined by instrumental measurement of structural and mechanical characteristics. A huge variety of food products prevents from providing any universal recommendations for choosing the method for estimating the rheological characteristics of dispersed food systems in which these properties change during production, storage, packaging, transportation, etc.

The rheological parameters of food systems are among the most important physicochemical characteristics that determine the role of the thickening agent used in food products. Rheological parameters were estimated from the yield point, which was determined on a rotational viscometer, by varying the type of the gelling agent, the degree of fineness of the berries, and the presence of various sugars and solvents.

The rheological tests were conducted for systems with the following ratio of components:

- 0.16 wt % of a thickening agent (sodium alginate; chitosan; blueberry, lingonberry, and cranberry pectin);
- 35.44 wt % of a solvent (milk with a weight fraction of fat of 1.5%, curd whey, and distilled water);
-64.4 wt % of sugar (saccharose, fructose, and sorbite) with allowance for the solid content.

The experimental data were used to plot flow curves in the γ/τ coordinates (strain rate gradient/shear stress) for systems based on water, milk, and whey; the curves are shown in Figs. 3–5.

All the flow curves in the figures deviate from a straight line; hence, the food systems corresponding to these curves are non-Newtonian (quasi-viscous) fluids and belong to structured disperse systems; their viscous properties depend on the composition and velocity gradient.

The rheograms confirm the non-Newtonian flow,

which is characterized by a disproportional decrease in viscosity with increasing shear rate.

Flow curves were plotted for all the systems according to the derived values of shear stress and viscosity: logarithmic dependences of apparent viscosity η on shear stress τ (Figs. 6–8).

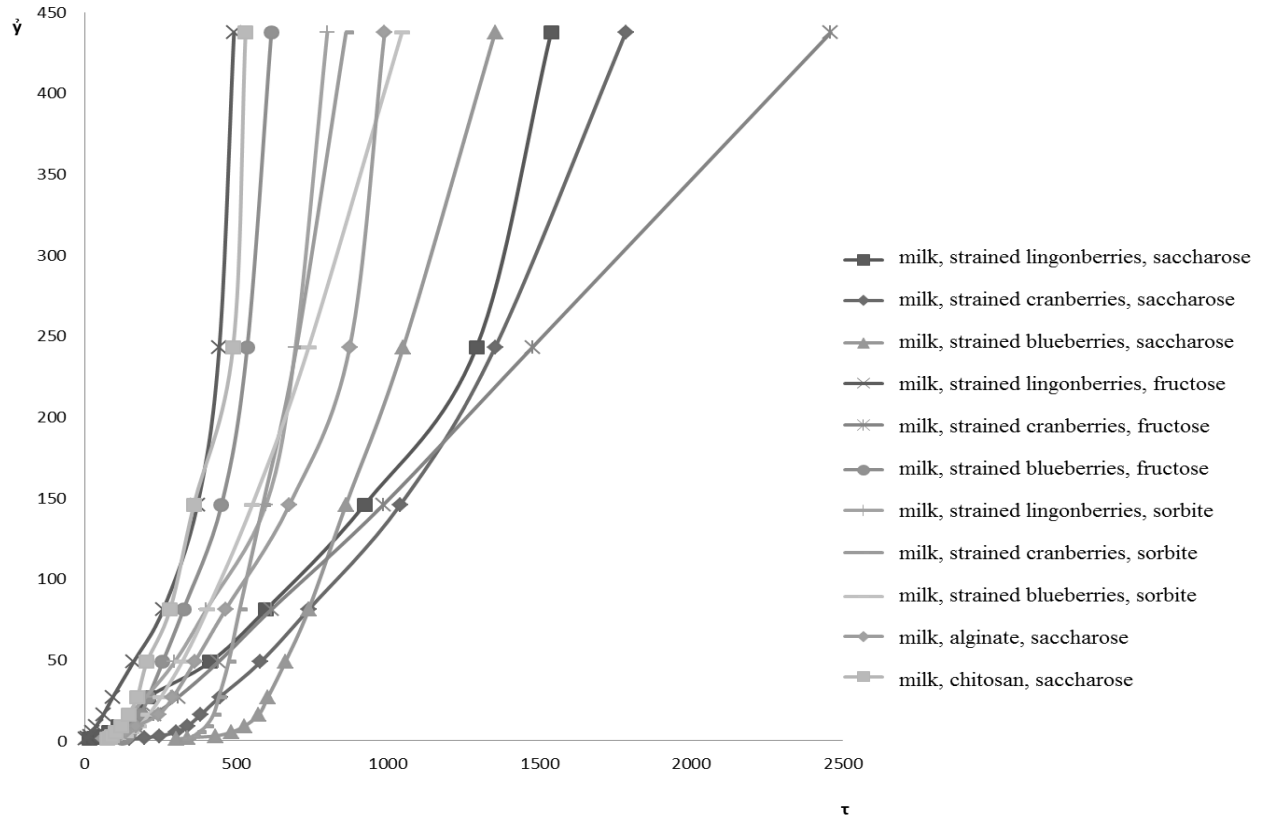


Fig. 3. Flow curves of the studied systems based on milk.

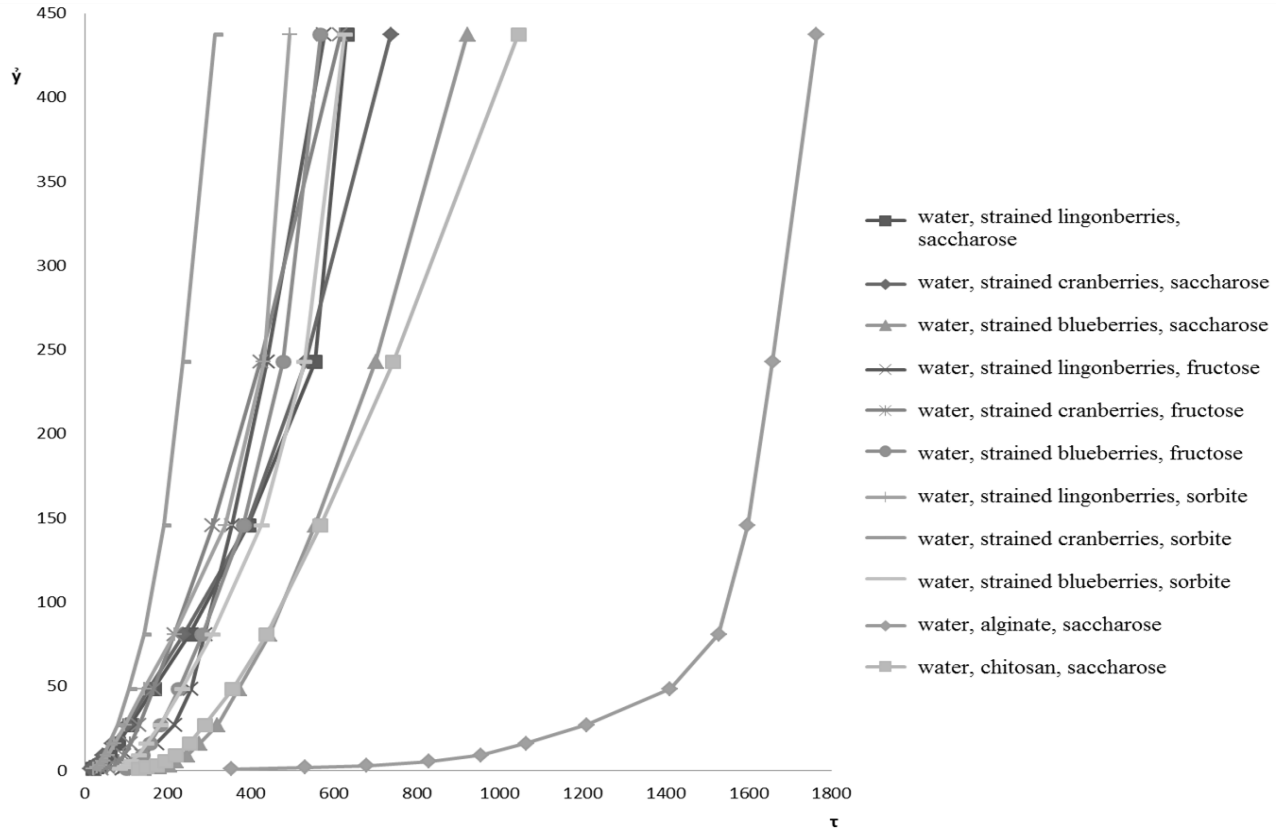


Fig. 4. Flow curves of the studied systems based on water.

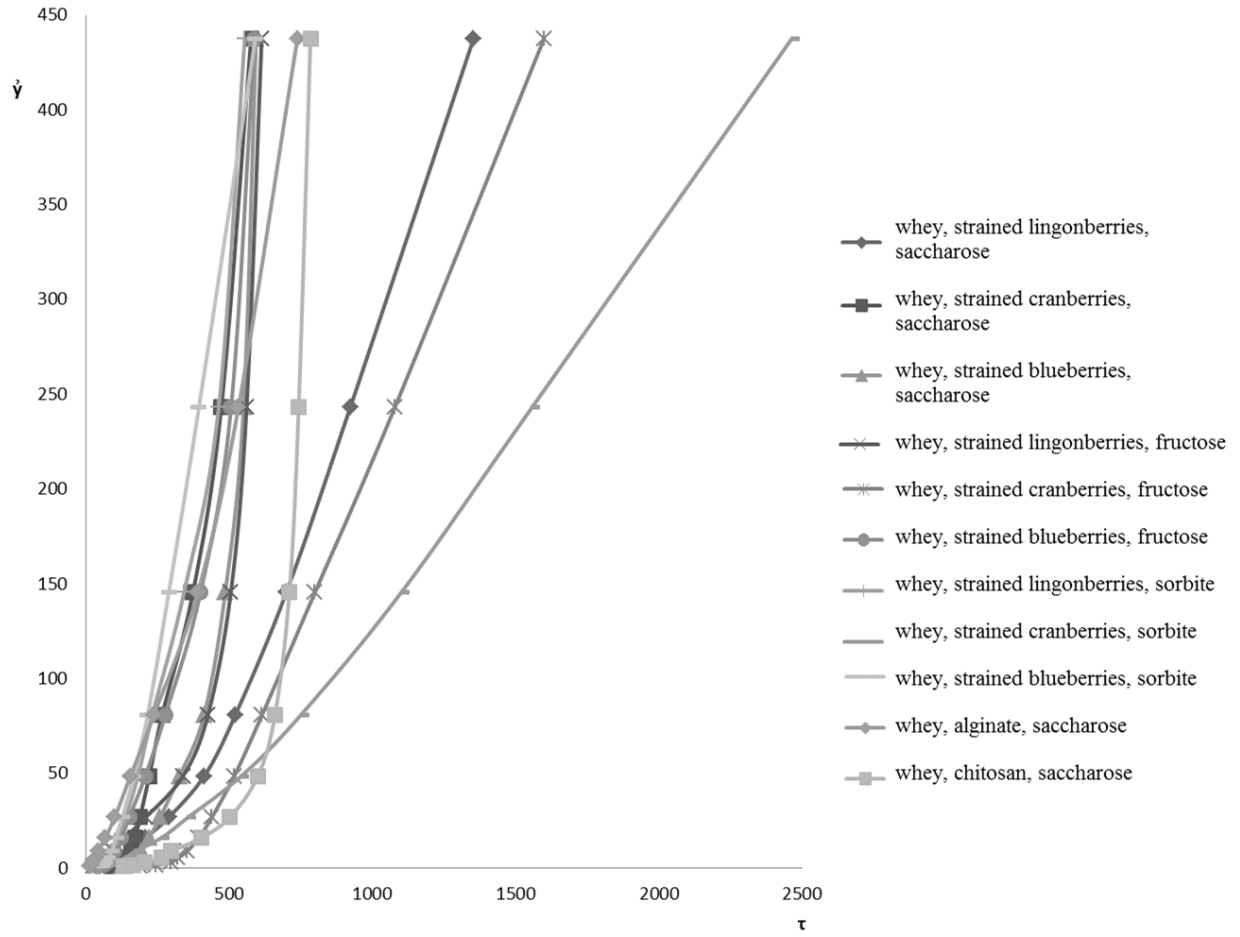


Fig. 5. Flow curves of the studied systems based on whey.

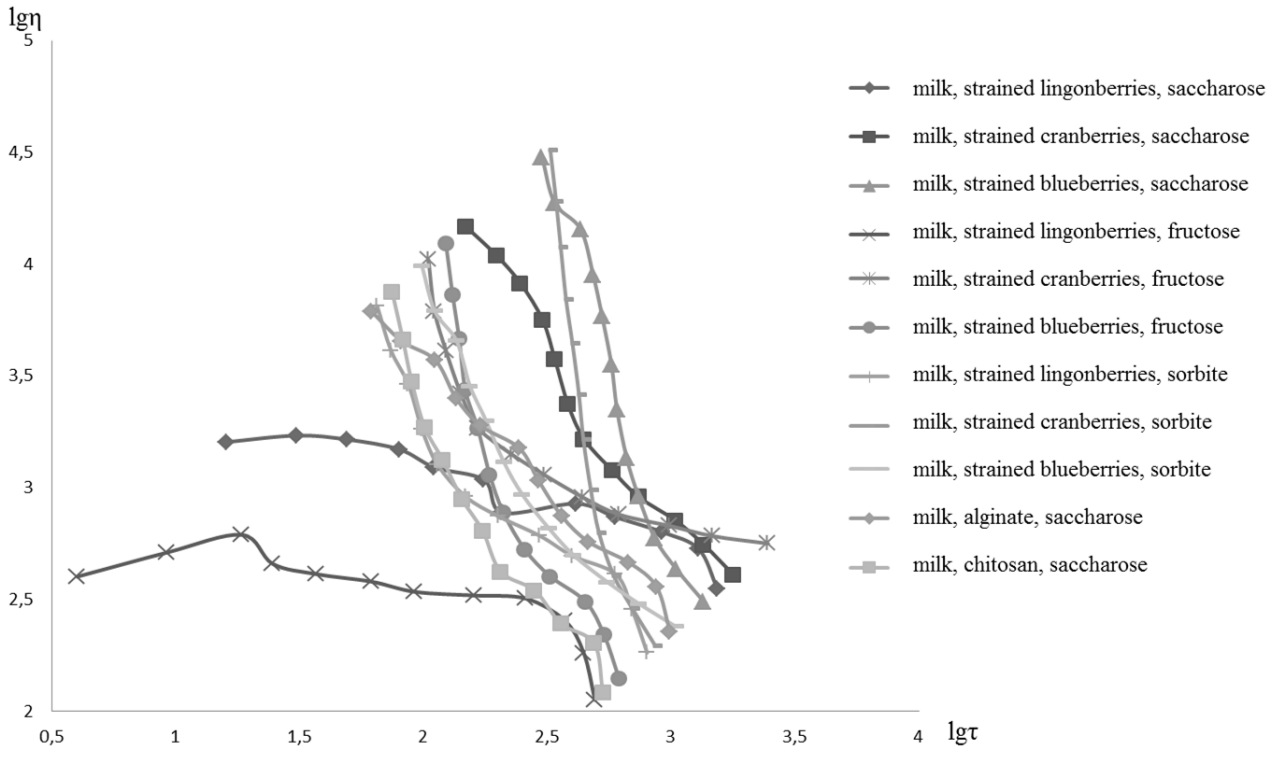


Fig. 6. Logarithmic dependence of apparent viscosity η on shear stress τ for the studied systems based on milk.

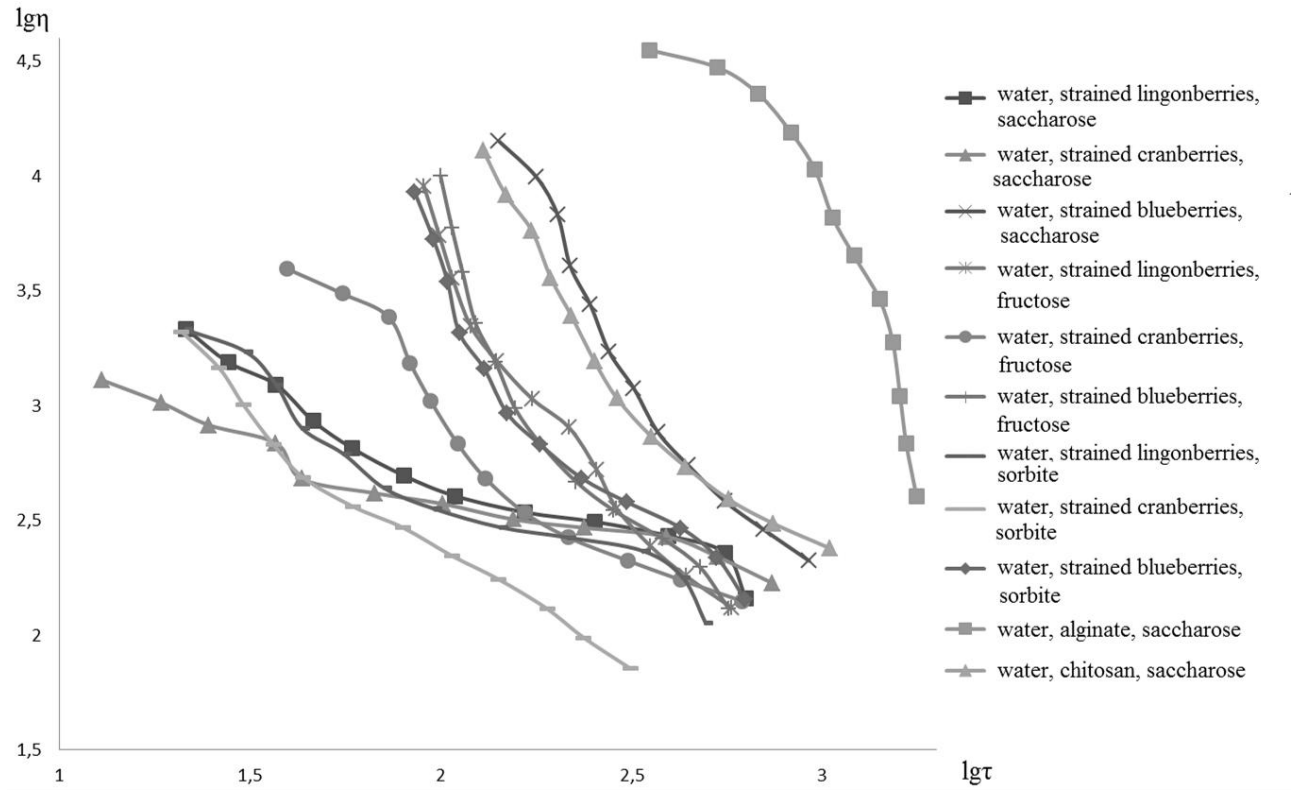


Fig. 7. Logarithmic dependence of apparent viscosity η on shear stress τ for the studied systems based on water.

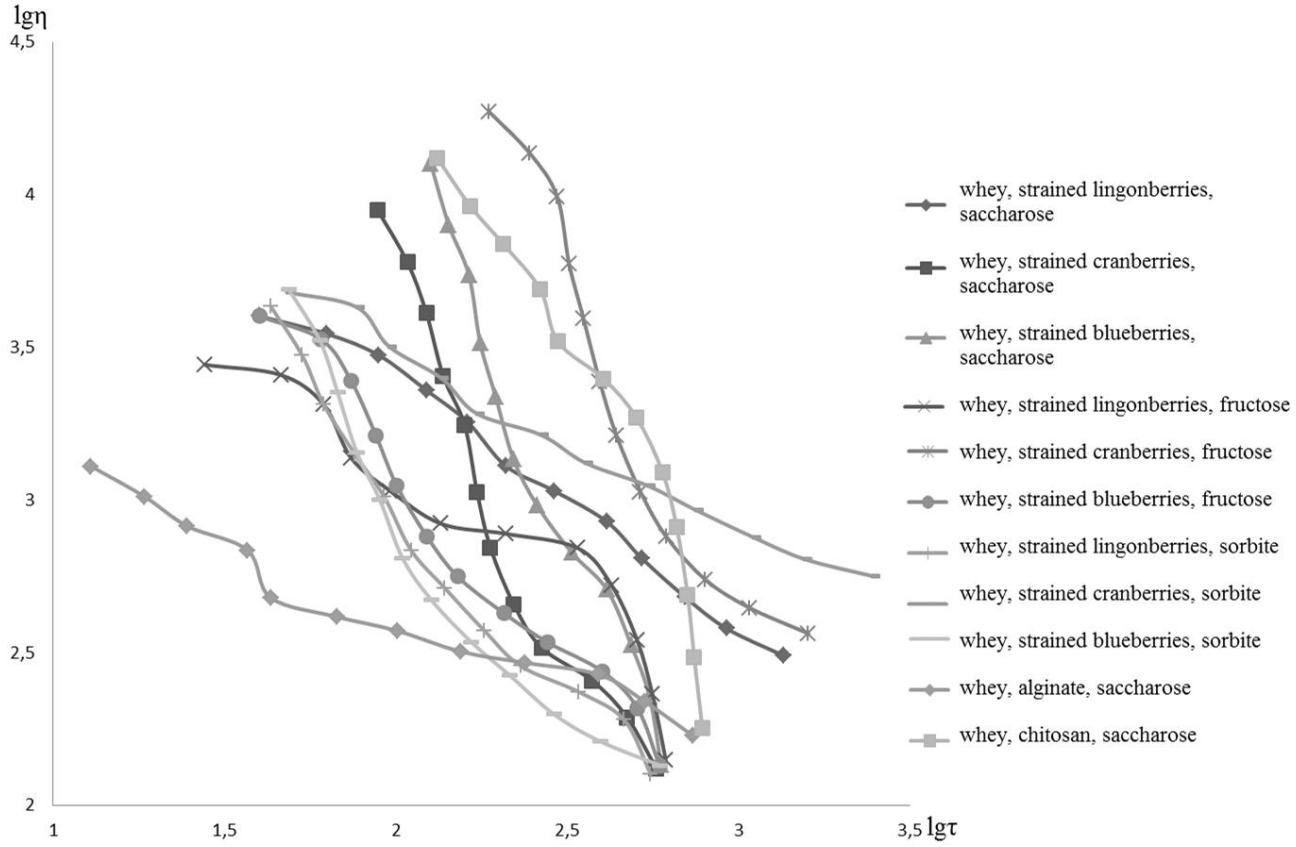


Fig. 8. Logarithmic dependence of apparent viscosity η on shear stress τ for the studied systems based on whey.

To describe the flow curves of the studied samples, the Ostwald–de Waele equation was used:

$$\tau = K \cdot \gamma^n, \qquad \lg\tau = n \cdot \lg\gamma + \lg K,$$

where τ is the shear stress, Pa; K is the flow consistency index (a measure of viscosity of the fluid); γ is the shear rate gradient, s^{-1}; and n is the flow behavior index (characterizes the degree of rheological difference between the studied product and a Newtonian fluid).

To find indices K and n in the Ostwald–de Waele equation, plots in the $\lg \tau = f(\lg \gamma)$ logarithmic coordinates were constructed. In all the cases, the rheological curves are fairly well approximated by a linear function.

RESULTS AND DISCUSSION

(1) Physicochemical studies of blueberries, lingonberries, and cranberries as a source of pectic substances have been conducted. The biochemical analysis of blueberries, lingonberries, and cranberries has revealed that the weight fraction of moisture is 86.5, 87, and 89.5%, respectively; the weight fraction of solids is 13.5, 13, and 10.5%, respectively; the titratable acidity corresponds to 2, 2.16, and 2.76%; the concentration of nitrate ions does not exceed 0.5 mmol/dm^3.

(2) Crystallized pectin in the form of a light brown powder has been isolated from blueberries, lingonberries, and cranberries. Quantitative analysis of the pectic substances has shown that the weight fraction of water-soluble pectin is 0.78% in blueberries, 0.77% in lingonberries, and 0.465% in cranberries; the amount of water-insoluble pectin (protopectin) is 0.52, 0.13, and 0.255%, respectively. The total amount of pectic substances is 1.3% in blueberries, 0.9% in lingonberries, and 0.69% in cranberries. The resulting pectins have the following degree of etherification: 86.2, 63.4, and 75.9% in blueberries, lingonberries, and cranberries, respectively.

(3) The effect of various solvents and sugars on the rheological properties of food systems containing pectic substances has been studied. It has been found that the pectic substances of wild-growing blueberries, lingonberries, and cranberries are fairly effective. High-etherified pectic substances contribute to the strengthening of the structure of the food system because they are stabilized in a gel owing to the combination of hydrophobic interactions and hydrogen bonds.

The use of pectic substances as gelling agents and various dehydrating agents—saccharose, sorbite, and fructose—has revealed that saccharose is the best dehydrating agent. The addition of saccharose contributes to the formation of hydrogen bonds, which leads to the binding of the solvent, the stabilization of hydrophobic interactions, and, as a consequence, to an increase in the viscosity and the strengthening of the gel structure. Therefore, the use of sugar substitutes in combination with pectin-containing feedstocks significantly decreases the viscosity of the finished product and, hence, its shelf life and quality.

Gels based on fructose and sorbite exhibit a lower apparent viscosity than gels based on saccharose. Therefore, the gel structure can be strengthened by increasing the dose of pectin. In the case of replacing saccharose with other sugars or sugar substitutes, it is necessary to increase their solubility and crystallizability [3, 16].

The action of thickening agents in milk solutions is enhanced compared to solutions based on whey; in turn, the action of these agents in whey-based solutions is more intense than in water solutions. A considerable increase in viscosity occurs owing to the formation of associates of the gelling agent with macromolecular components of the food system. Thus, the efficiency of thickening agents is determined not only by the structural features of their molecules, but also by the composition of the food raw material.

(4) A comparative estimation of the gelling ability of the pectic substances contained in blueberries, lingonberries, and cranberries and in chitosan and sodium alginate gelling agents has been conducted. The viscous properties increase in the series: cranberry pectin < lingonberry pectin < blueberry pectin < sodium alginate < chitosan.

(5) The complexing ability of pectin with respect to copper and iron ions has been studied. A comparative estimation of the binding ability of pectin and casein with respect to metal ions has been conducted. The complexing ability depends on the concentration of both pectin and casein. It has been revealed that casein exhibits a lower complexing ability with respect to iron ions than pectin. It has been found that the complexing properties of pectin vary with its concentration: the more dilute the solution, the higher the complexing ability of pectin. The highest complexing ability of pectin of 80 000 *mgCu^{2+}/g of pectin* is observed at a pectin concentration of 0.005 mg/cm^3.

REFERENCES

1. Edelev, D.A., Bril', D.E., Demidova, T.I., et al., Spetsializirovannye pishchevye produkty s sorbtsionnymi i nutritivnymi svoistvami (Specialized food products with sorption and nutritive properties), *Pishchevaya Promyshlennost'* (*Food Industry*), 2012, no. 6, pp. 59–61.
2. Gorbachev, M.G. and Demidova, T.I., Spetsializirovannye produkty so svoistvami enterosorbenta (Specialized products with enterosorbent properties), *Pishchevaya Promyshlennost'* (*Food Industry*), 2012, no. 5, pp. 36–38.
3. Donchenko, L.V. and Firsov, G.G., *Pektin: osnovnye svoistva, proizvodstvo i primenenie: monografiya* (Pectin: Basic Properties, Production, and Application: A Monograph), Moscow: DeLi Print, 2007.
4. Dunets, E.G. and Zaiko, G.M., Detoksikatsionnye svoistva pektinosoderzhashchikh pishchevykh system (Detoxifying properties of pectin-containing food systems), *Izvestiya Vysshikh Uchebnykh Zavedenii, Pishchevaya Tekhnologiya* (*Bulletin of Higher Educational Institutions, Food Technology*), 2008, no. 4, pp. 43–44.

5. Lisitskaya, K.V., Torkova, A.A., Nikolaev, I.V., et al., Sravnitel'nyi analiz antioksidantnykh svoistv pektinov iz razlichnogo rastitel'nogo syr'ya (A comparative analysis of the antioxidant properties of pectins from various plant raw materials), *Pishchevaya Promyshlennost'* (*Food Industry*), 2012, no. 12, pp. 64–66.
6. Piskureva, V.A., Gor'kova, I.V., Pavlovsksy, N.E., and Zhitnikova, V.S., Bakteritsidnye i kompleksoobrazuyushchie svoistva pektinov plodoovoshchnogo kontsentrata (Bactericidal and complexing properties of pectins of a fruit-and-vegetable concentrate), *Pishchevaya Promyshlennost'* (*Food Industry*), 2009, no. 6, pp. 50–51.
7. Demidova, T.I. and Shevernitskaya, O.N., Prioritetnye napravleniya razvitiya tekhnologii proizvodstva pektinsoderzhashchikh produktov (Priority directions of development of technologies for the manufacture of pectin-containing products), *Pishchevaya Promyshlennost'* (*Food Industry*), 2010, no. 9, pp. 18–19.
8. Dvoenosova, P.A., Doronin, A.F., and Izotova, T.I., Funktsional'nye pektinsoderzhashchie produkty pri sindrome kishechnoi nedostatochnosti (Functional pectin-containing products in the case of intestinal failure syndrome), *Pishchevaya Promyshlennost'* (*Food Industry*), 2009, no. 6, pp. 54–55.
9. Tikhomirova, N.A., Zaiko, G.M., Korneva, O.A., et al., Napitki funktsional'nogo naznacheniya na osnove soevogo moloka i pektinsoderzhashchego dikorastushchego syr'ya (Functional beverage foods based on soy milk and pectin-containing wild-growing plant raw materials), *Izvestiya Vysshikh Uchebnykh Zavedenii, Pishchevaya Tekhnologiya* (*Bulletin of Higher Educational Institutions, Food Technology*), 2012, no. 2–3, pp. 95–96.
10. Tolkunova, N.N., Zhitnikova, V.S., and Kozicheva, M.A., Issledovanie aktivnosti rastvorimogo pektina po stepeni eterifikatsii i kompleksoobrazuyuschei sposobnosti (Analysis of the activity of soluble pectin from the degree of etherification and complexing ability), *Tekhnologiya i Tovarovedenie Innovatsionnykh Pishchevykh Produktov* (*Technology and Merchandizing of Innovation Food Products*), 2012, no. 2, pp. 24–28.
11. Khrundin, D.V., Romanova, N.K., and Reshetnik, O.A., Vliyanie di- i trikarbonovykh kislot na sorbtsiyu ionov medi vysokoeterifitsirovannymi pektinami (Effect of di- and tricarboxylic acids on the sorption of copper ions by high-etherified pectins), *Izvestiya Vysshikh Uchebnykh Zavedenii, Pishchevaya Tekhnologiya* (*Bulletin of Higher Educational Institutions, Food Technology*), 2011, no. 4, pp. 22–23.
12. Il'ina, I.A., Sapelnikov, Yu.A., Mironova, O.P., and Zemskova, Z.G., Metodologicheskie osnovy protsessa kompleksoobrazovaniya pektinov (Methodological bases of the complexation of pectins), *Izvestiya Vysshikh Uchebnykh Zavedenii, Pishchevaya Tekhnologiya* (*Bulletin of Higher Educational Institutions, Food Technology*), 2003, no. 5/6, pp. 35–38.
13. Shamkova, N.T., Svyazyvayushchaya sposobnost' pektinsoderzhashchikh pishchevykh system (The binding ability of pectin-containing food systems), *Izvestiya Vysshikh Uchebnykh Zavedenii, Pishchevaya Tekhnologiya* (*Bulletin of Higher Educational Institutions, Food Technology*), 2006, no. 5, pp. 20–22.
14. Rumyantseva, G.N., Varfolomeeva, O.A., and Kindra, N.A., Sokhranenie zheliruyuschei sposobnosti pektina v protsesse ego vydeleniya iz yablochnykh vyzhimok (Preservation of the gelling ability of pectin during its isolation from apple pomace), *Khranenie i Pererabotka Sel'khozsyr'ya* (*Storage and Processing of Agricultural Raw Materials*), 2006, no. 7, pp. 25–28.
15. Shamkova, N.T., Svyazyvayushchaya sposobnost' model'nykh sistem pektin-belok-voda (The binding ability of pectin–protein–water model systems), *Izvestiya Vysshikh Uchebnykh Zavedenii, Pishchevaya Tekhnologiya* (*Bulletin of Higher Educational Institutions, Food Technology*), 2007, no. 1, pp. 19–21.
16. Magomedov, G.O., Magomedov, A.K., Miroshnikova, T.N., and Lobosova, L.A., Vliyanie fruktozy na studneobrazovanie pri proizvodstve zefira na pektine (Effect of fructose on the gelation in the production of marshmallow based on pectin), *Konditerskoe Proizvodstvo* (*Confectionery*), 2007, no. 2, pp. 31–33.

IMMOBILIZATION OF CHYMOTRYPSIN ON MAGNETIC Fe_3O_4 NANOPARTICLES

L. S. Dyshlyuk, M. V. Novoselova, and T. A. Rozalenok

Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
phone:/fax: +7 (3842) 39-05-37, e-mail: soldatovals1984@mail.ru

Received May 28, 2013; accepted in revised form September 5, 2013

Abstract: Modern methods of chemical modification of enzymes conferring increased catalytic activity and stability to these molecules have been considered. The advantages of using magnetic nanoparticles for the production of stable immobilized enzyme preparations are presented. Chymotrypsin immobilization on Fe_3O_4 nanoparticles modified with amino groups has been found to result in the incorporation of 88% of the enzyme into the solid phase. The change of the optimal pH and temperature ranges and an increase of stability of the immobilized chymotrypsin relatively to the respective characteristics of the native enzyme have been demonstrated.

Key words: catalytic activity, magnetic nanoparticles, chymotrypsin, glutaraldehyde method, immobilization

UDC 663.15:620.3
DOI 10.12737/2060

INTRODUCTION

The possibilities of application of enzymes, especially in medicine and food processing, have expanded significantly due to the recent advances in enzymology. This is due to the obvious advantages of enzymes over chemical catalysts, namely, selectivity and stereospecificity of action, the ability to attain high substrate conversion rates under relatively mild technological conditions, and safety for the environment and humans [1, 2].

Most enzymes used in food processing are present in food and are ingested with fresh fruit and vegetables, nuts, milk, and fermented and canned foods. The search for new applications of enzymes in various fields of biotechnology is currently going on. The main areas of research include modification of the properties of individual enzymes in order to increase their activity and reduce the cost of the end products, screening of novel enzyme-producing microorganisms, generation of novel recombinant enzymes with desired properties, the use of enzymatic reactions for the production of valuable food ingredients and bioactive substances, and the development of enzyme-based nanotechnology procedures for food processing [2].

The modern methods of enzyme modification confer increased resistance to a variety of chemicals and inhibitors, as well as to pH and temperature effects, to these molecules and allow for alteration of the pH optimum, substrate specificity, and binding properties of the enzymes. Moreover, the catalytic properties of enzymes and the preference of these biocatalysts towards certain metal cofactors can be regulated by modification [2].

Chemical modification of enzymes is among the most widely used methods [3, 4]. The chemical modification procedures must meet a number of requirements. Firstly, the method should employ non-hazardous chemical reagents, especially in case of enzymes subsequently used in food industry. Secondly, harsh conditions of modification leading to enzyme deterioration should be avoided. Thirdly, separation of the modified enzymes from the reaction medium by relatively simple and inexpensive methods should be possible. Finally, the use of the modified enzymes should be cost-effective [4].

The use of non-polar reaction media is an example of chemical modification of enzymes [5]. The resulting reduction of water activity in the reaction system leads to substantial changes of the properties of enzymes, namely, the reaction is shifted towards synthesis, the thermal stability and the storage stability of the enzyme increase, the enzyme acquires an ability to catalyze novel reactions not occurring in an aqueous environment and retains activity in organic solvents at a temperature above 100°C. This method of chemical modification is applicable for such enzymes as lipase, chymotrypsin, trypsin, subtilisin, thermolysin, polyphenol oxidase, glucoamylase, papain, and chymosin.

Research on biological methods of enzyme modification is an actively developing area of enzymology. Protein engineering is an especially promising approach. The methods of protein engineering based on information on the relationship between amino acid sequence, three-dimensional structure, and catalytic activity of enzymes allow for successful modification of enzymes resulting in improvement of their technological characteristics [6, 7]. Substitution of certain amino acid residues in the enzyme molecule is a widely used method.

Substitution of amino acid residues in the enzyme molecules can be used to alter the substrate specificity of these biocatalysts [8]. For example, the ratio of cellobiohydrolase activity towards soluble and insoluble substrates can be altered by replacing the external aromatic amino acid residues which bind to the end of the polysaccharide molecule and direct it into the active site. Resistance of the enzyme to high temperature and extreme pH values is achieved by replacing pairs of amino acid residues located close to each other in the tertiary structure of the enzyme in order to create additional non-covalent hydrophobic bonds, salt bridges, or cova-

lent S-S bonds conferring higher general stability to the globular enzyme molecule.

Chymotrypsin is an enzyme that belongs to the hydrolase class and preferentially cleaves bonds formed by aromatic amino acids (tyrosine, phenylalanine, and tryptophan) in proteins and peptides. Chymotrypsin is among the enzymes most frequently used in various areas of biotechnology, including food industry. Diverse approaches to the regulation of the catalytic activity of this enzyme, including changing the degree of hydration in systems of hydrated reverse micelles formed by surfactants [9], use of nonpolar solvents, adsorption, retention in porous matrices, covalent binding, electrochemical polymerization, etc., have been reported. However, these approaches mostly result in conformational changes manifested as reduced proteolytic activity and increased Michaelis constant of chymotrypsin.

Enzyme immobilization on various organic and inorganic carriers, both natural and synthetic, is among the approaches most widely used in studies described above. This method is universal due to the simplicity of the techniques applied, uniform distribution of the enzyme in the bulk carrier, stability of the immobilized preparations obtained, and good reproducibility of their analytic characteristics. Moreover, attachment of enzymes to solid supports allows for a significant improvement of the mechanical properties of enzyme preparations.

A large variety of carriers for the immobilization of biomolecules is available; however, researchers have been recently showing considerable interest towards nanoparticle carriers [10, 11] due to the changes in a range of fundamental properties of matter upon the transition to the nano-sized state. The large surface of the nanosized objects provides for the predominance of surface phenomena and therefore it is one of the main factors determining the physical properties of nanoscale objects. Since the size of nanoparticles is comparable to those of cells, viruses, proteins, and DNA, these particles can approach biological objects, bind to them, and interact with them.

Nanotechnology is currently recognized as a priority direction of research worldwide since it integrates the cutting-edge achievements of physics, chemistry, and biology. The potential of this discipline is enormous, and its realization affects every aspect of human life. Development of approaches to precise manipulation of the functioning of living systems on the subcellular level is a key issue of modern research in the field of nanobiotechnology.

According to the general concepts outlined above, the aims of the present study included optimization of the conditions for the production of a highly efficient and stable chymotrypsin preparation with Fe_3O_4 nanoparticles as the carrier, as well as the assessment of the perspectives and reasonability of its use in various areas of biotechnology.

RESULTS AND DISCUSSION

The presence of a sufficient amount of active groups on the surface of the carrier is a prerequisite for successful enzyme immobilization. However, the surface of Fe_3O_4 nanoparticles is virtually devoid of reactive groups which could be used for covalent binding of the enzyme, and therefore chemical modification prior to enzyme immobilization is necessary. Various methods for modifying Fe_3O_4 nanoparticles have been reported, including alkylation, acetylation, amination, silanization, coating with polysaccharides or polyaniline, etc. Covalent immobilization of chymotrypsin can be performed using glutaraldehyde which joins the amino groups on the surface of the modified nanoparticles to the amino groups of the enzyme, acting as a spacer.

Crystalline α-chymotrypsin (Sigma, the United States) with an activity of 40–60 U/mg, 70% glutaraldehyde and L-tyrosine (99.9 %) from Sigma-Aldrich-Louis (the United States), sodium caseinate (protein content 92% by mass) kindly provided by the Belka company (Moscow, Russia), and other reagents of the "chemically pure" grade manufactured in Russia were used in the present work.

Fe_3O_4 nanoparticles were prepared using the procedure of Massart [12] by treating a 1:2 mixture of ferrous and ferric chloride solutions (concentration 0.25 M) with a 30% (m/m) NH_4OH solution at room temperature. The resulting precipitate was heated to 80ºC for 30 minutes under constant stirring and washed several times with water and ethanol, and afterwards the Fe_3O_4 nanoparticles were separated from the supernatant using a magnetic separator and dried under vacuum at a temperature above 70ºC [15]. The reaction of Fe_3O_4 formation can be schematically represented as follows:

$$FeCl_2 + 2FeCl_3 + 8NH_3 \cdot H_2O \rightarrow Fe_3O_4 + 8NH_4Cl + 4H_2O$$

Surface modification of the nanoparticles was performed by incubating them with urea and dimethylformamide at a temperature of 300°C. Urea was used for the amination of the surface. Afterwards, the Fe_3O_4 nanoparticles modified with amino groups were washed with distilled water several times for complete removal of impurities and dried at 25°C. The washed nanoparticles were equilibrated with 0.1 M phosphate buffer (pH 7.4) and incubated with 25% glutaraldehyde for two hours at room temperature with vigorous stirring. After the incubation the nanoparticles were washed several times with distilled water.

Chymotrypsin immobilization was carried out by adding the enzyme to a suspension of modified nanoparticles in 0.1 M phosphate buffer (pH 7.4). The enzyme which was non-covalently bound to the particles was removed by three washes with distilled water, a 1% aqueous solution of sodium chloride, and a 1% alcohol solution of sodium chloride. The immobilized chymotrypsin preparation was dried at 4°C and stored as a suspension in 0.1 M phosphate buffer at 4°C.

Estimation of the amount of the immobilized enzyme was based on the difference between the protein concentrations in the reaction mixture before and after incubation of chymotrypsin with Fe_3O_4 nanoparticles. The protein content was determined by the Dumas method based on measuring the thermal conductivity of molecular nitrogen generated after combustion of the test sample at a temperature of about 1000ºC in an atmosphere of oxygen and subsequent complete reduction of nitrogen oxides with copper using the protein nitrogen analyzer RAPID N Cube (Elementar, Germany).

The proteolytic activity of the native and immobilized enzyme was assessed according to the modified method of Anson (GOST (State Standard) 20264.2-88 "Enzyme preparations. Methods for assaying proteolytic activity") based on the determination of the rate of enzymatic hydrolysis of sodium caseinate by the enzyme preparation investigated and colorimetric quantitation of the peptides and amino acids formed using the Folin reagent.

The results of the measurements were processed using the methods of mathematical statistics.

The experiment showed that 88% of chymotrypsin was incorporated in the solid phase after six hours of incubation with Fe_3O_4 nanoparticles modified with amino groups in the presence of glutaraldehyde (Fig. 1). The reaction conditions (pH, temperature, composition of the buffer for immobilization, etc.) were optimized in order to attain maximal proteolytic activity of the immobilized enzyme.

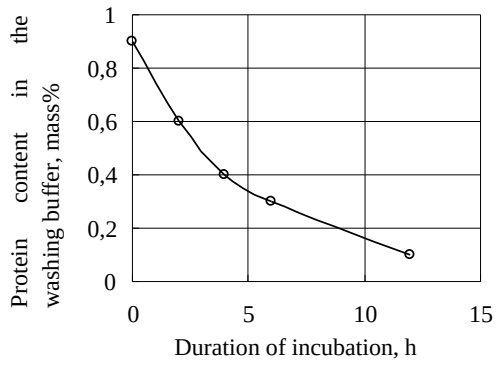


Fig. 1. The dependence of the mass concentration of the protein in the contact solution on the duration of immobilization.

The optimal pH and temperature ranges are important characteristics of the catalytic activity of enzymes. Therefore, investigation of the effect of chymotrypsin immobilization on the optimal functioning conditions of the enzyme was one of the objectives of the present study. Both native and immobilized enzyme were shown to exhibit maximum catalytic activity at pH 8.0 (Fig. 2), but the optimal pH range was much broader for the immobilized enzyme than for native chymotrypsin. The optimal temperature for the immobilized chymotrypsin differed from that of the native chymotrypsin by 5ºC (Fig. 3).

Investigation of the stability of the immobilized enzyme preparation suspended in 0.1 M phosphate buffer (pH 7.4) and stored in a dry, dark place at a temperature of (4 ± 2)ºC (Fig. 4) revealed the decrease of catalytic activity of both native and immobilized chymotrypsin during storage. Native chymotrypsin completely lost activity after 20 days of storage, while chymotrypsin attached to nanoparticles modified with amino groups retained 20% of the catalytic activity after 25 days of storage. Therefore, immobilization of chymotrypsin on Fe_3O_4 nanoparticles results in stabilization of the enzyme.

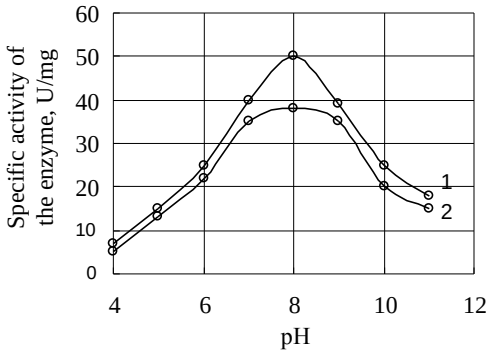


Fig. 2. The pH dependence of the specific activity of chymotrypsin: 1 – native enzyme; 2 – immobilized enzyme.

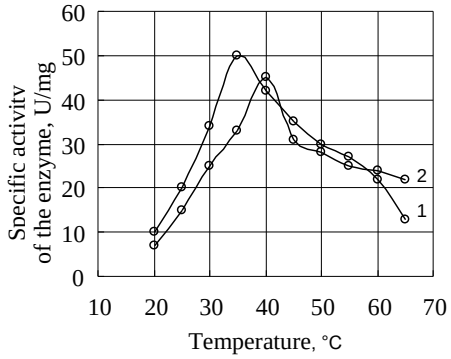


Fig. 3. The temperature dependence of the specific activity of chymotrypsin: 1 – native enzyme; 2 – immobilized enzyme.

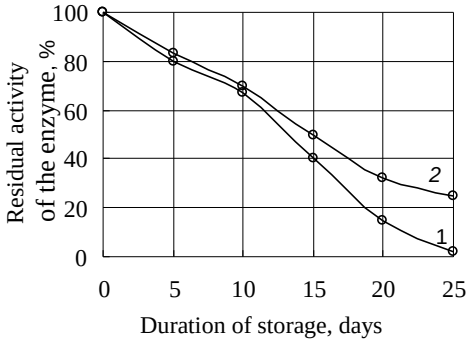


Fig. 4. The dependence of the catalytic activity of chymotrypsin on the duration of storage:1 – native enzyme; 2 – immobilized enzyme.

CONCLUSIONS

Covalent binding of chymotrypsin to surface-modified nanoparticles allows for the production of immobilized preparations possessing high activity and stability; these preparations can be used in different

fields of biotechnology. Besides, the properties of enzyme preparations can be regulated in a controllable manner according to the specific biotechnological tasks by varying the conditions of immobilization.

REFERENCES

1. Volova, T.G., *Biotekhnologiya* (Biotechnology), Novosibirsk: Izdatel'stvo SO RAN, 1999.
2. *Biotekhnologiya* (Biotechnology), Higgins, I., Best, D., and Jones, G., Eds., Moscow: Mir, 1988.
3. Eggins, B., *Khimicheskie i biologicheskie sensory* (Chemical sensors and biosensors), Moscow: Tekhnosfera, 2005, no. 3, pp. 25–31.
4. Gracheva, I.M., *Tekhnologiya fermentnykh preparatov* (Technology of enzyme preparations), Moscow: Agropromizdat, 1985.
5. Antipova, L.V., *Primenenie fermentov v pererabotke vtorichnogo molochnogo syr'ya* (The use of enzymes in the processing of secondary raw materials of dairy origin), Moscow: AgroNIITEIMMP, 1992.
6. Andreeva, I.N., Pantyukhin, A.V., and Sysuev, B.B. *Immobilizatsiya fermentov i drugikh biologicheski aktivnykh soedinenii. Uchebnoe posobie* (Immobilization of enzymes and other biologically active substances. Study guide), Pyatigorsk: Pyatigorskaya Gosudarstvennaya Farmatsevticheskaya Akademiya, 2001.
7. Berezin, I.V. *Biotekhnologiya. Uchebnoe posobie dlya vuzov. T. 7. Immobilizovannye fermenty* (Biotechnology. Student training manual. Vol.7. Immobilized enzymes), Moscow: Vysshaya Shkola, 1987.
8. Berezin, I.V., Klesov, A.A., and Shvyadas, V.K., *Inzhenernaya enzimologiya* (Engineering enzymology), Moscow: 1987.
9. Dixon, M. and Webb, E., *Fermenty. Tom 1,2* (Enzymes. Vols. 1, 2), Moscow: Mir, 1982.
10. Berry, C., and Curtis, A., Functionalization of magnetic nanoparticles for applications in biomedicine, *J. Phys. D. Appl. Phys.*, 2003, vol. 36, pp. R198–R206.
11. Gu, H., Xu, K., and Xu, C., Biofunctional magnetic nanoparticles for protein separation and pathogen detection, *J. of the American Chemical Society Chem. Commun.*, 2006, pp. 941–949.
12. Massart, R., Preparation of aqueous magnetic liquids in alkaline and acidic media, *IEEE Trans. Magn.*, 1981, vol. 17, no. 2, pp. 1247–1248.

The present study was carried out within the framework of the Federal Target Program "Research and research-pedagogic personnel of innovative Russia" for 2009–2013

ECONOMY OF THE AGROINDUSTRIAL COMPLEX

SPECIFIC DEVELOPMENT OF THE BAKING INDUSTRY IN KEMEROVO OBLAST

A. N. Kiryukhina* and N. M. Guk**

Kemerovo Institute of Food Science and Technology,
bul'v. Stroitelei 47, Kemerovo, 650056 Russia,
*phone:/fax: +7 (3842) 39-68-62, e-mail: lika1455@rambler.ru
**phone:/fax: +7 (3842) 39-68-63, e-mail: gukkkk@nextmail.ru

Received January 11, 2013; accepted in revised form March 4, 2013

Abstract: The article shows the dynamics of the output of baked goods over the past five years in Russia and in the Siberian Federal District. The main trends in the development of the baking branch of the food industry of Kemerovo oblast are specified. Factors that determine the dynamics of baked goods production in the Kuznetsk Basin are considered. Practices of transfer from administrative bread price regulation to targeted subsidies for the least protected strata are shown. Analytical results of the dynamics and structure of the assortment of bread baked by large and medium-sized bakeries of the oblast are presented. Ways of improving the current efficiency of the baking branch of the food industry of Kemerovo oblast are determined.

Key words: baking industry, baked products, production output, «social» bread, assortment

UDC 664.66(571.17)
DOI 10.12737/2061

INTRODUCTION

Bread is a brilliant invention of humankind. Breadstuffs are major human food products, regularly bought by everyone and everywhere. The supply of the most affordable foodstuff to all strata depends on how efficiently this branch functions and develops.

Daily bread consumption in various countries varies from 150 to 500 g per capita. In Russia the average bread consumption rate is up to 330 g a day. The diagram of breadstuff consumption in some countries of the European Union is given in Fig. 1. *[Consumption of certain foodstuffs per inhabitant. Eurostat. Search database. http://www.eurostat.ee.europa.eu]*

According to the Russian Federal Statistics Service (RFSS), the average bread consumption in Russia in 2007–2011 stayed at 119 kg/yr per capita; i.e., we are close to countries with average consumption in this respect (Austria, France, and Lithuania, 116.4, 116.7, and 120.8 kg/yr, respectively).

As for the number of businesses, production output, and importance of product output, the baking branch is one of the most crucial areas in Russia's food industry. The special literature and mass media regularly publish analytical materials on Russia's bread market, its characteristics, problems, and trends in its development.

The activity reports of President of the Russian Union of Bakers A.P. Kosovan periodically contain the assessment of the state of affairs in the industry. The Russian baking industry is notable for the leadership of large units, companies, and holding companies, whose share in the total output of baked goods is about 80%.

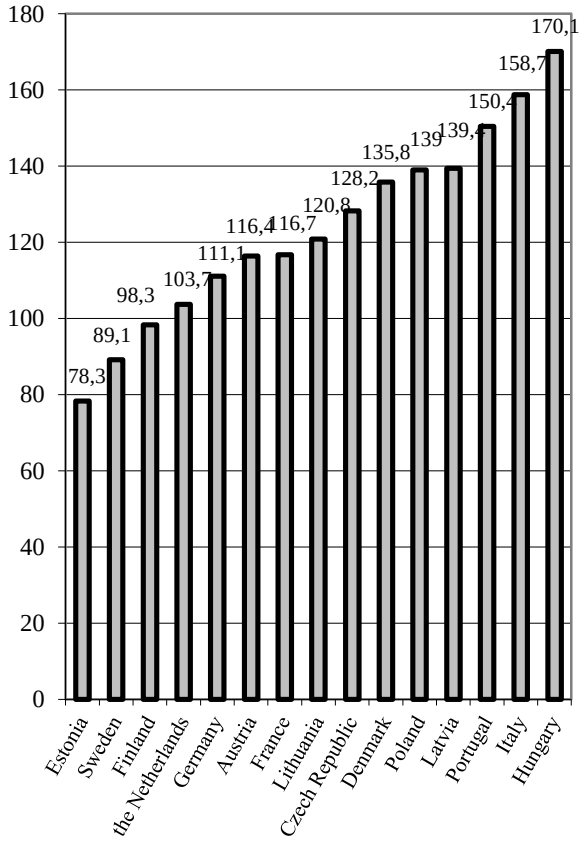


Fig. 1. Consumption of breadstuffs in EU countries in 2006–2009 (kg/yr per capita).

Among the industry's most serious problems are its obsolete physical infrastructure, low financial stability, shortage of skilled personnel due low labor remuneration, the constant growth of fuel and energy tariffs, and unpredictable fluctuations in flour prices. The lasting practice of administrative intervention in economic activities aggravates the financial position of bakeries. The stand of the Russian Union of Bakers is that low-income strata must have targeted government support. Thus far the economy of bakeries is forced to be guided by the income level of the poorest strata, scaffolding the excessive financial load of the unalterably low incomes of its employees and turning Russia's bread baking into the «face of the country's poverty» [1].

The situation in the bread market was described in a number of articles by N.T. Chubenko, the chief specialist of the State Research Institute of the Baking Industry, Russian Academy of Agricultural Sciences, published in the industrial journal *Khlebopechenie Rossii* (Russia's Bread Baking). The author cited data that, in 2010, large bakeries used only 40% of their capacity. At the same time, they produced more than 70% of saleable products. Only 882 such units remained in 2010 (out of the total 12 300 active bakeries); in ten years the number of operating bakeries decreased by more than 1.5 times [2].

The output of baked goods in the country is steadily decreasing. According to expert forecasts, this market will lose 2% in natural units on average a year and by 2014 it will not exceed 6.5 mln. t. The official statistical data in Table 1, which characterize the change in the output of baked goods in Russia and in the Siberian Federal District (SFD), show that the production dynamics of bakeries in Kemerovo oblast has a trajectory different from the overall vector in Russia and in the Siberian Federal District.

Table 1 Dynamics of the output of baked goods

Regions	Output, thou. tons					Growth rate, %			
	2007	2008	2009	2010	2011	2008/ 2007	2009/2008	2010/2009	2011/2010
Russia	7759	7483.3	7191.4	7119	6977	96.4	96.1	99.0	98.3
Siberian FD	1031	978.8	928.6	896.3	877.5	94.9	94.9	96.5	97.9
Altai Republic	6.8	6.5	6.8	7.2	6.9	95.6	104.6	105.9	96.3
Buryat Republic	39.8	38.3	34.7	28.9	32.2	96.2	90.6	83.3	111.5
Tyva Republic	27.3	27.5	27.7	27.8	27.8	100.7	100.7	100.4	100.0
Khakassia	19.1	25.2	19.4	18.2	18.9	131.9	77.0	93.8	104.3
Altai krai	118.3	120.3	120.1	122.0	116.6	101.7	99.8	101.6	95.8
Transbaikal krai	78.3	52.2	46.6	39.7	38.3	66.7	89.3	85.2	96.4
Krasnoyarsk krai	113.9	108.3	93.1	100.2	93.0	95.1	86.0	107.6	92.8
Irkutsk oblast	117.7	105.9	96.0	90.5	84.2	90.0	90.7	94.3	93.1
Kemerovo oblast	183.3	181.4	185.2	184.7	185	99.0	102.1	99.7	100.2
Novosibirsk oblast	181.1	169.0	158.8	134.1	140.3	93.3	94.0	84.4	104.7
Omsk oblast	113.9	115.8	115.0	114.7	110.4	101.7	99.3	99.7	96.2
Tomsk oblast	31.7	28.2	25.3	28.4	23.6	89.0	89.7	112.3	83.4

Among the constituent members of the Siberian Federal District, Kemerovo oblast holds the first place in the output of bread and baked goods, followed, respectively, by Novosibirsk oblast and Altai krai. Despite the overall decrease in production in the Siberian Federal District, the output of baked goods in Kemerovo oblast increased to 185 000 t/yr between 2007 and 2011.

Research objective. To identify factors that affect the output dynamics of baked goods in Kemerovo oblast.

Research subject and methods. The subject of research in this article is large and medium-sized bread-baking units in Kemerovo oblast. The research was conducted using the traditional methods of economic analysis: abstract–logical, statistical, comparative, graphic, economic–mathematical, etc., as well as statistical and analytical materials of the RFSS, the RFSS branch for Kemerovo oblast, the financial statements and annual reports of large and medium-sized baking units of Kemerovo oblast, and the statistical data of various Russian and foreign web sites.

Results and discussion. The transformation of the Russian economy has led to great changes in the output of the baking industry both in the Russian Federation and in Kemerovo oblast. According to the RFSS data for Kemerovo oblast, in the early 2000s the baking industry of the Kuznetsk Basin witnessed a shard decline in the output of baked products (Fig. 2).

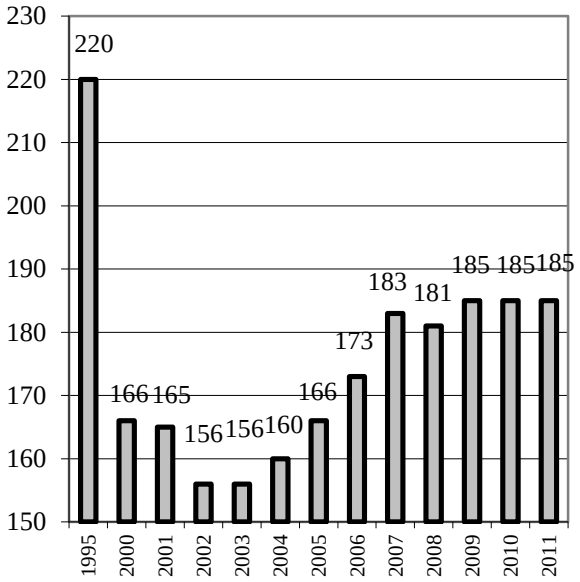
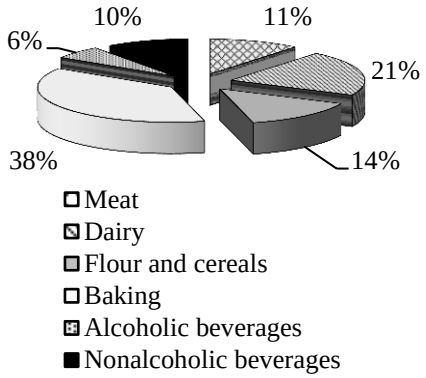


Fig. 2. Production of bread and baked goods in Kemerovo oblast (thou. tons).

The situation started to improve in 2004, and already in 2005 the output reached the level of 2000. Then we can trace the annual growth of this indicator: the output was 166 000 t in 2005, 173 000 t in 2006, and 183 000 t in 2007 (i.e., the growth rate was more than 10% in three years) with an upward trend in the subsequent years, whereas the production of bread and baked goods in Russia in 2005–2009 dropped to the level of 1998. In 2009 the production of bread and baked goods in Kemerovo oblast rose to 185 000 t, reaching 119% of the 2003 level. Between 2010 and 2011 the output remained at the 2009 level.

The baking branch holds a key place in providing the people of Kemerovo oblast with staple foodstuffs. Its share in the structure of the oblast's food industry is 38% of the total food output (Fig. 3).

In 2011 the baking branch was 67% bread and baked goods, whose share in the total output continued to increase annually unlike candy and pasta manufacture (Table 2).



* Liquid volumes were converted to weight indices taking into account the beverage density gradations according to the density table.

Fig. 3. Structure of the food industry of Kemerovo oblast.

Table 2 Structure of the baling branch of Kemerovo oblast

Indices	Unit.	2007	2008	2009	2010	2011	Growth rate, %			
							2008/ 2007	2009/ 2008	2010/ 2009	2011/ 2010
Bread and baked goods	thou. t	183.3	181.4	185.2	184.7	185.0	99.0	102.1	99.7	100.2
% of the total		63.1	63.4	66.2	66.8	67.3	100.5	104.4	100.9	100.7
Confectionery products	thou. t	101	99.1	89.2	87.2	86.1	98.1	90.0	97.8	98.7
% of the total		34.7	34.6	31.9	31.6	31.3	99.7	92.2	99.1	99.1
Pasta	thou. t	6.4	5.6	5.4	4.4	3.9	87.5	96.4	81.5	88.6
% of the total		2.2	2.0	1.9	1.6	1.4	90.9	95.0	84.2	87.5
Total	thou. t	290.7	286.1	279.8	276.3	275.0	98.4	97.8	98.7	99.5

The main industrial potential of the baking branch in Kemerovo oblast is concentrated in the cities of Kemerovo, Novokuznetsk, Leninsk-Kuznetsk, Yurga, Prokopyevsk , and Mezhdurechensk. At present there are 10 large and medium-sized bakeries and more than 370 small-sized units. Over the past years, we have seen a reduction in the output of baked goods at large and medium-sized units (Fig. 4).

In 2011 large and medium-sized bakeries produced about 57% of the total bread output in the oblast (Table. 3), which was decreasing with each year. Consequently, small businesses, whose share is about 43% of the total output of baked goods, are playing an increasingly important role in providing the people of Kemerovo oblast with various freshly baked goods.

Table 3 Baked goods output by Kemerovo oblast's bakeries in 2011

Bakeries	Output, tons	Percentage of the total, %
Total, including	185 000	100.0
large and medium-sized	105 820	57.2
small	79 180	42.8

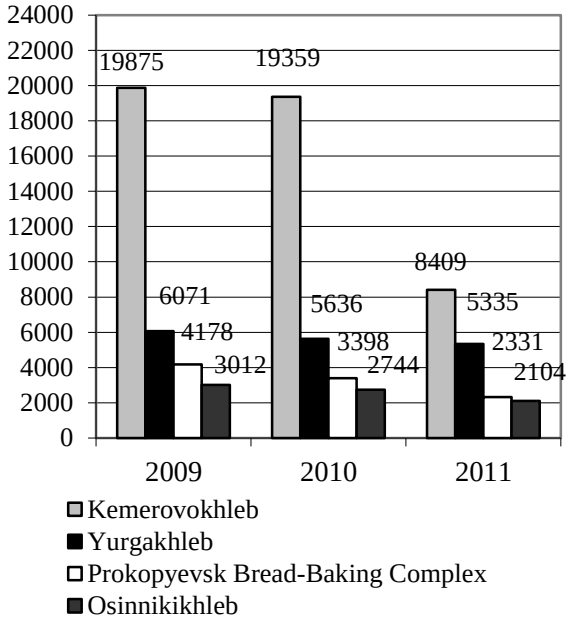


Fig. 4. Bread and baked goods output dynamics (by the example of individual units in Kemerovo oblast), tons.

Small businesses are not at all inferior to large bakeries in their quality of baked goods, since they run new equipment, although their share of manual labor is higher. The main advantages of such units are mobility, considerable room for private orders, and always fresh products. The number of minibakeries in Kemerovo oblast is growing mainly due to the increased number of hypermarkets, supermarkets, and chain stores, almost each of which has its own bakery.

By the results of the financial and economic analysis of the existing bakeries and trends in the Kuznetsk Basin's bread market, we may conclude that in recent years the increased output of baked goods in Kemerovo oblast has been maintained by small businesses.

Referring to the baking practices in Europe, we may note that state-of-the-art minibakeries play a significant role there, providing certain countries with up to 80% of the baked goods consumed (Fig. 5).

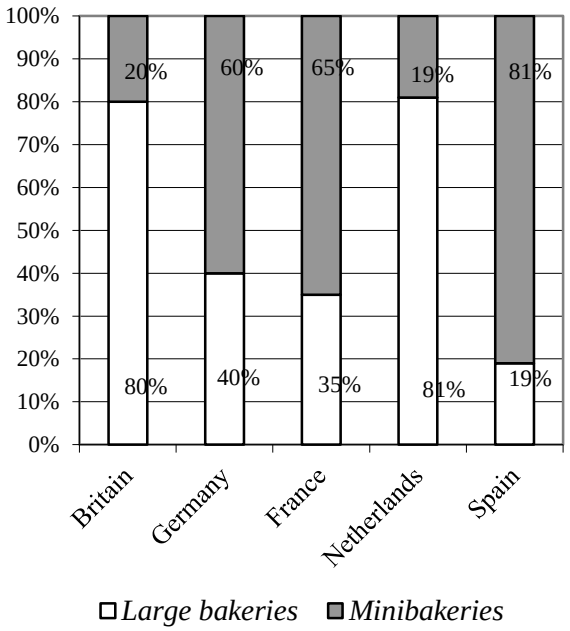


Fig. 5. Production of baked goods by large and small businesses in some European countries.

Minibakeries that produce bread and confectionery products locally are the main players in the national markets of such countries as Germany, France, and Spain. In Britain and the Netherlands, the share of small businesses in the bread market does not exceed 20%. On average, the bread market in Europe in 2010, according to the International Association of Plant Bakers (AIBI), was represented by large bakeries (45%) and minibakeries (55%) *[http://www.bakersfederation.org.uk/the-bread-indust-ry/industry-facts/european-bread-market.html]*.

In European countries, minibakeries are also in high demand among the owners of supermarkets, hypermarkets, restaurants, and cafes. For example, in Britain out of the 20% of the bread market served by minibakeries, 17% are minibakeries at retail outlets (Table 4) *[http://www.bakersfederation.org.uk/the-bread-industry/about-the-bread-industry.html]*.

Table 4 Shares of various producers in the British bread market

Units	% of output In monetary terms	% of output in natural units
Large bakeries	78	80
Minibakeries	22	20
Including minibakeries at retail outlets	17	17
Total	100	100

In addition to the above factor (serious competition on the part of minibakeries), a significant drop in bread output by large and medium-sized units in 2011 was preconditioned by retracting the production of «social» bread in Kemerovo oblast, which was baked from 1999 through May 1, 2011. This measure of social support was necessary for the majority of people under the unstable conditions of the oblast's socioeconomic development, such as significant price growth, unpaid wages, and the stingy pensions of the overwhelming majority of retirees. Within this program, the oblast administration subsidized bread producers with flour from the governor's grain reserve fund. The difference between purchasing and subsidized prices was finally compensated for from the oblast budget. The producers, in turn, set underrated prices on more than 60% of their bread products. The activity of the grain reserve fund, which is formed of grain bought from the local farmers at the highest prices in Siberia, is regulated by Kemerovo oblast's Law no. 23-OZ of April 8, 2008, which says that the governor's grain fund was created for the stable provision of the people of Kemerovo oblast with baked goods in line with the food security policy of our region [3]. This instrument charged bakeries with the production of «social» bread, i.e., first-quality wheat bread weighing 500 g at a selling price of 7.27 rubles. Trade networks could impose only a 10% markup. Therefore, bread produced in the Kuznetsk Basin was the cheapest compared with other regions of Russia (8 rubles a loaf for several years).

By 2011 the level of personal incomes rose, and the economic situation improved, but the bakeries were still under the administrative pressure. A situation began to form in which bread was baked for the needy but consumed by all. Numerous resolutions were issued to increase the output of «social» bread to avoid the shortage of this product among the needy. As a result, the share of «social» bread baked at the oblast's large bakeries reached 70% in their total output.

Surveys conducted in November 2010 by OOO Sotsioservis showed that «social» bread was increasingly misused: 10% of the oblast population used it to feed livestock and poultry; 13% of the population said that they witnessed such misuses; and 23% of the people witnessed such cases several times. People who really needed cheap bread were often left without this support. All this drew indignation in the majority of people *[http://www.kuzzbas.ru/ more.phpUID=16980]*.

The estimation of the demand for this product, based on the number of the needy citizens, for whom this «so-

cial» bread was produced, and the rational norms of daily bread consumption [4] showed that its amount necessary for the people of Kemerovo oblast was 3800 t a year (Table 5).

Table 5 Estimation of the necessary amount of «social» bread (2010)

Indices	Value
Population of Kemerovo, thou. people	521.2
Share of the needy, %	12.1
Number of the needy, thou. people	63.06
Bread consumption norm per capita, g/day including wheat bread rye bread	330 165 165
Necessary wheat bred output, t/day	10.4
Necessary rye bread output, t/yr	3798.1

However, OAO Kemerovokhleb alone produced 10 700 t of «social» bread in 2010 on the condition that this product was not profitable. In addition, the financial support of bakeries as quotas to purchase 70% of their flour demand at preferential prices did not solve the existing problem. Estimations based on the calculation for this product showed that increased quotas would reduce the production cost insignificantly, and even the use of 100% of the reserve fund flour would not allow the bakers to have a positive financial result from the production of «social» bread.

Such measures incessantly required additional costs and affected very negatively the size of the oblast budget. Therefore, in April 2011, the administration of Kemerovo oblast made a decision to render a targeted cash aid of 60 rubles a month per capita from the oblast budget starting from May 1, instead of subsidizing the bakers for «social» bread, to the following categories of citizens:

- nonworking pensioners with incomes below the minimum subsistence level (4013 rubles);
- families with children under 16 years with incomes below the minimum subsistence level for each family member (5335 rubles);
- large needy families that bring up three and more children, including those from 16 to 18 years of age, with incomes below the minimum subsistence level (5335 rubles);
- students of higher educational establishments and technical colleges from needy families (with incomes below the minimum subsistence level of 5335 rubles for each family member); and
- Students of elementary vocational schools from needy families.

Overall, from May 1, 2011, almost 180 000 people in the oblast started to receive additional cash payments. The amount of the payment was determined on the basis of the medical consumption rate (10 loafs per capita), the average bread price in Russia (14 rubles), and the cost of «social» bread in the Kuznetsk Basin (8 rubles). The difference in prices (6 rubles) multiplied by the consumption rate amounted to the additional payment of 60 rubles a month per capita *[http://kemerovo.edinros.ru /theme/450.html]*.

In our opinion, this approach to the solution of the «social» bread problem, first, affected positively the oblast budget. In 2010, 390 million rubles were allocated from the oblast budget to produce «social» bread and in 2011, on the basis of the targeted aid (60 rubles a month per capita) and the number of people who qualified for it (180 000), 129.6 million rubles; i.e., the economic effect of this measure was significant. Second, this approach made it possible to increase the size of profit received by the bakeries (Table 6) and contributed to the improvement of the industry's competitiveness. Thus, first-quality wheat bread became a profitable product for large bakeries for the first time in many years. However, the oblast administration put this bread on the list of socially crucial products so that bakers do not raise its price to the limit in their chase for profit.

Comparing the average consumer price for the group «First- and second-quality bread and baked goods from wheat flour,» the lion's share of which is first-quality wheat bread, we see that in Kemerovo oblast it is still the lowest among the constituent members of the Siberian Federal District (Table 7) [http://www.gks.ru.].

Table 6 Financial results of first-quality wheat bread production (by the example of the Prokopyevsk Bread-Baking Complex)

Indices	Before May 01, 2011		From May 01, 2011	
	per 1 t	per 1 loaf	per 1 t	per 1 loaf
Production cost, rubles	16850.1	8.41	20165.3	10.08
Including reserve fund flour, rubles	2382.8	1.19	-	-
flour at market prices, rubles	2442.0	1.22	8140	4.07
Wholesale price, rubles	13261.0	6.63	23120.0	11.56
Gross profit, rubles	- 3589.1	- 1.78	2954.7	1.48
Profitability, %	-	-	14.7	14.7
Output	3692 t	7 384 000 loafs	3692 t	7 384 000 loafs
Gross profit, thou. rubles	- 13 205.9		10 908.7	

Table 7 Average consumer prices of bread and goods baked from first- and second-quality wheat flour, rubles/kg

SFD constituent members	April 2011	April 2012
Siberian Federal District:	28.75	30.19
Altai Republic	28.03	27.66
Buryat republic	30.8	31.42
Tyva Republic	29.44	29.26
Republic of Khakassia	26.79	26.03
Altai krai	28.5	28.38
Transbaikal krai	32.95	34.62
Krasnoyarsk krai	30.36	30.72
Irkutsk oblast	34.29	34.83
Kemerovo oblast	16.21	24.18
Omsk oblast	26.87	26.35
Novosibirsk oblast	37.2	37.62
Tomsk oblast	29.14	27.72

The positive result of this decision was also the reduction of cheap bread purchases for misuse, which

guaranteed to the needy strata the use of this affordable product. Overall, this approach has led to the increased efficiency of the existing system; it was opportune and popular among the market players; and it had never been used by other constituent members of the Russian Federation; therefore, the practice of shifting to subsidies for the needy from bread price regulation should be adopted by other regions of Russia.

In recent years, significant changes have occurred in the assortment structure of baked goods produced by the large and medium-sized units of Kemerovo oblast. The assortment structure and dynamics in 2009–2011 are given in Table 8. A positive trend in the assortment development is a stable decrease in the output of wheat bread varieties, including top-quality flour, most poor in micronutrients, while the output share of rye breads has started to increase, which is in line with the directions of assortment development and the norms of healthy diet.

Table 8 Output structure of baked goods (by the example of individual units of Kemerovo oblast)

Name	2009		2010		2011		2009		2010		2011	
	t	%	t	%	t	%	t	%	t	%	t	%
	Prokopyevsk Bread-Baking Complex						Leninsk-Kuznetsk Bakery					
1) rye and rye–wheat bread	489.5	11.7	467.2	13.7	456	19.6	2555	17.5	3084	21.7	4391	30.5
2) wheat bread of all varieties	3299.5	79.0	2526.8	74.4	1568	67.3	7636	52.3	7813	55.0	6898	48.0
3) baked products	326.1	7.8	276.5	8.1	255.8	10.9	2942	20.2	2770	19.5	2791	19.4
4) other products	63.2	1.5	127.5	3.8	51.2	2.2	1458	10.0	543	3.8	299	2.1
Total	4178.3	100.0	3398	100.0	2331	100	14591	100.0	14210	100.0	14379	100.0

At present the oblast's bakeries put great efforts in the production of bread and baked goods enriched with vitamins and minerals, i.e., dietary and preventive products. New varieties of bread and baked goods are being developed and introduced. Along with the production of the usual varieties, the bakeries of Kemerovo oblast have mastered breads with various additives: walnut, sesame, cereal flakes, laminaria, dry green mixes, vegetables, etc. (more than 50 new bread varieties). A lot of attention is paid to the production of so-called «healthy bread,» Doctor's crisp breads and crisp breads with walnuts, onions, and greens. More often than not you can find products in special packaging.

New bread recipes have been developed by local technologists jointly with associates of the Kemerovo Institute of Food Science and Technology and the Kemerovo State Medical Academy. Branded bread varieties such as Prazhskii, Boyarskii, Venskii, Fitness with Raisins from Sprouted Seeds, Finnish Wheat Crusts, Golden with Coriander, Flax Seed, Village Bread, and Russian Bread have already gained acceptance from authoritative world-scale specialists and have become laureates of multiple exhibitions and competitions.

The bakers of Kemerovo oblast produce bread prepared on yeast, nonyeasted bread, bread on hoppy starters, bread with seed additives, and bread with malt extracts. There are also bread varieties that do not harm even people who suffer from diabetes or obesity, for example, coarse-grained bread. It is slowly digested by the organism and does not increase the blood sugar level; therefore, the pancreas has no need to work to the maximum (the varieties Otrubnoi, Izobilie, and Linga). Bread from kibbled grain is even more useful: it contains all the valuable ingredients that whole grain has, like coarse fiber that inhibits diabetes.

The above indicates that the bakers of the Kuznetsk Basin are actively developing enriched bread varieties for dietary nutrition and for functional purposes envisaged in the Food Security Doctrine, approved by a decree of the Russian president. This is confirmed by the output of diabetic bread varieties, and Kemerovo oblast is the leader among the Russian regions in their production (Table 9) [5].

Table 9 Output of diabetic breads in Russia

Regions	Produced in 2009, tons
Russia's total, including federal districts:	1058
Central FD	179
Northwestern FD	164
Southern FD	37
Northern Caucasus FD	10
Volga FD	240
Ural FD	103
Siberian FD, including:	325
- Transbaikal krai	35
- Irkutsk oblast	4
- Kemerovo oblast	286

At present, the production of functional foodstuffs is a major and dynamically developing branch of the food industry in countries that uphold a healthy life style. European bread-baking experience shows that European countries have been able to reach a stable situation in the consumption of baked goods by making the consumer consider bread as an element of healthy nutrition and not just as a source of calories. Since the 1990s Europeans have been displaying the growing demand for products with improved taste properties, which leads to considerable shifts in the assortment of foodstuffs: the reduction of the share of traditional mass bread varieties and the increase in functional and premium breads. New bread varieties appear whose recipes include wheat, rye, or oat boltings, whole unground grain, oat and buckwheat flour, vegetable and fruit additives, and other components. As a result, the share of healthy bread in the total bread output has increased by 68% in Britain and by two times in Germany in recent years [6]. Figure 6 shows the assortment structure of the German bread

market, and we can see that the share of healthy bread in its total supply reached 63% in 2010 *[The German Bakery Industry. Facts and Figures 2011. http//www.iba.de/]*.

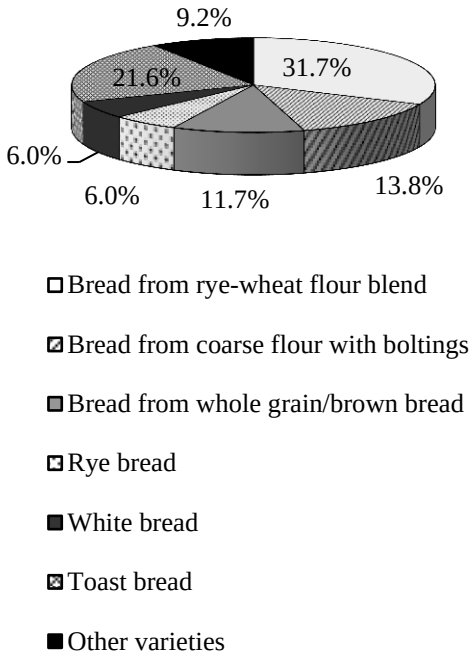


Fig. 6. Assortment structure of Germany's bread market.

The government policies of European countries aimed at increasing consumer awareness of the composition of foodstuffs contributes to the increased consumption of functional baked goods. Printing this information on packaging, the producer informs the consumer of the effects of the raw materials of which products are made and prevents the development of certain diseases. The approvals of their products by various medical associations and societies (of endocrinologists, cardiologists, and other medical specialists) become important for European producers. In particular, the special Food Information Regulation to inform people about the ingredients of foodstuffs is in effect in the European Union (). Using this information, each consumer can choose products taking into account individual preferences [7].

Today fewer and fewer consumers buy what they are offered, and more and more of them seek and buy products that meet their preferences. A survey conducted in the United States to reveal the consumer behavior of the generation born in 1980–2000 has shown that people consciously choose what they buy; they are very sensitive to advanced brands, value highly the freedom of choice on the retention of requirements on healthy foodstuffs, and pay great attention to the format of presentation of product information, including the Internet *[Generation Y is driving food trends, says report. Food navigator-USA.com. 19.01.2009]*.

Taking into account the current negative operating conditions (reduced production output and profitability, operating losses) and the European experience, the large and medium-sized bakeries in Kemerovo oblast must base their current activities on constant concern for the stable quality of products and for the renewal of their assortment in line with the requirements of the science of nutrition. In choosing their assortments, the bakeries must be geared to marketing research and, more importantly, to scientific recommendations. To enhance the food ration and to prevent diseases, the oblast's bakery plants must continue to increase the output of rye bread varieties, master the output of special dietary varieties with bread-enriching additives. Competent control of quality and assortment as the chief factors of production increase will affect positively the sales and competitiveness of the bakeries of the Kuznetsk Basin.

Taking into account the opinion of many specialists of the baking industry that, to improve the efficiency of this industry, urgent measures such as the elimination of administrative regulation of bread prices, the transfer to the targeted subsidizing of the least protected strata, the increased output of dietary and functional baked goods, and the production of bread from rye flour, as well as the development of the infrastructure and interaction between science, higher educational establishments, and business are necessary, we may state that today the baking branch of the food industry of Kemerovo oblast is developing in the right direction.

REFERENCES

1. Khlebopekarnaya promyshlennost' Rossii v 2008–2012. Otchetnyi doklad X s"ezdu Rossiiskogo Soyuza pekarei prezidenta RSP A.P. Kosovana (Russia's Baking Industry in 2008–2012. Activity Report to X Congress of the Russian Union of Bakers by RUB President A.P. Kosovan), *Khlebopechenie Rossii (Russia's Bread Baking)*, 2012, No. 5, pp. 6–9.
2. Chubenko, N.T., O situatsii na rynke khleba (Situation in the Bread Market), *Khlebopechenie Rossii (Russia's Bread Baking)*, 2012, No. 1, pp. 6–8.
3. *Zakon Kemerovskoi oblasti ot 8 aprelya 2008 g. No. 23-OZ «O regional'nom fonde zerna Kemerovskoi oblasti»* (Law of Kemerovo oblast of April 8, 2008, No. 23-OZ, On the Regional Grain Fund of Kemerovo Oblast).
4. *Zdorov'e naseleniya i okruzhayushchaya sreda g. Kemerovo*: *informatsionnyi sbornik (Human Health and the Environment of Kemerovo City: An Information Booklet)*, Kemerovo, 2008.
5. Chubenko, N.T. and Shlelenko, L.A., Razvitie assortimenta khlebobulochnykh izdelii v Rossiiskoi Federatsii (Developing the assortment of baked goods in the Russian Federation), *Khlebopechenie Rossii (Russia's Bread Baking)*, 2011, No. 1, pp. 8–10.

6. Alferov. A., Rynok khleba i khlebobulochnykh izdelii: realii, perspektivy, tendentsii razvitiya (The market of bread and baked goods: Realities, prospects, and trends of development), *Khleboprodukty (Bread products)*, 2009, No. 2, pp. 60–61.
7. Shaposhnikov, I.I., Kontseptsiya i prognoz razvitiya khlebopekarnoi promyshlennosti Rossii v 2011–2015 gg. (Concept and forecast of development of Russia's baking industry in 2011–2015), *Khlebopechenie Rossii (Russia's Bread Baking)*, 2011, No. 1, pp. 4–7.