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DEVELOPMENT OF THE BIOTECHNOLOGY OF *STREPTOCOCCUS THERMOPHILUS* BACTERIA AS PRODUCERS OF EXOPOLYSACCHARIDES

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Abstract. On carrying out a screening, phage-resistant strains of *Streptococcus thermophilus* have been selected. As producers of exopolysaccharides, they can be used to form the texture of fermented milk and improve the rheological properties of fermented milk products in a natural, effective, and safe way. It has been suggested to use analysis of the capsule-formation of strains as an express method to evaluate the rheological properties. The technology of the bacterial concentrate *Iprovit – Streptococcus thermophilus* with phage-resistant producers of exopolysaccharides has been developed. The following technological modes were used: growing in a growth medium with the ratio of carbohydrates to soluble nitrogen C:N=3.5; biomass stabilisation (10% inulin protective medium, freezing at minus 60±1°C for 16±2 h, drying for 24±2 h to 32±1°C, moisture content in the preparation not more than 5%). These modes allowed obtaining 7.83±0.02 g of bacterial concentrate from 1 dm^3 of the growth medium. Depending on the strain of *Streptococcus thermophilus*, the dry bacterial concentrate contained 4.4×10^{11}–1.1×10^{11} CFU/g of active microbiota. A technique has been developed that allows improving the rheological properties of fermented milk products by blending bacterial preparations to produce various products with concentrate of the *Streptococcus thermophilus* strain. The optimal formulations of the mixtures have been selected. This has made it possible to modify the rheological characteristics of fermented products without using other thickeners. The effective viscosity of the products obtained has been increased by 13.3–54.3%, depending on the formulation of the mixture. The biotechnology of the bacterial concentrate *Iprovit – Streptococcus thermophilus* was implemented at the State Research Enterprise of the Institute of Food Resources, National Academy of Agricultural Sciences of Ukraine. Manufacture of the concentrate is regulated by the regulatory documents developed and approved: TU 15.5-00419880-100:2010 "Dry and liquid fermenting cultures. Specifications," Technological Instructions for production of the fermented cultures *IPROVIT* for TU 15.5-00419880-100:2010 "Dry and liquid fermenting cultures. Specifications." The novelty of the technological solutions was confirmed by Copyright Registration Certificate No. 51033.

Key words: *Streptococcus thermophilus*, phage-resistant strains, producers, exopolysaccharides, capsules, effective viscosity, biotechnology.

Introduction. Formulation of the problem

Streptococcus thermophilus is a component of starter cultures to produce fermented baked milk and yoghurt, a variety of cheeses and dairy drinks. The important technological properties of this species of lactic acid bacteria (LAB) include moderate acid formation (acid production limit not more than 100-120°T), poor

proteolytic activity, and the ability to affect the consistency, viscosity of a fermentation product due to biosynthesis of various exopolysaccharides (EPS). It is known that some strains of the species *S. thermophilus*, while developing in milk, are able to produce EPS of two types: free extracellular slime, and capsular polysaccharides tightly attached to the cell surface. The presence of a capsule and its size are important features that allow a bacterial cell to survive in an adverse environment. Besides, they belong to the most important features that determine the rheological characteristics of fermented milk products [1]. EPS manifest their moisture-retaining ability and increase the viscosity of the soluble phase of the product. It has been shown that the presence of EPS, especially of capsular ones, provokes gelation at higher pH values. Such early aggregation processes allow more structural changes to take place during the formation of gel, which results in a more compact structure. EPS products are much more resistant to mechanical impacts, so it is recommended to use exopolysaccharide-producing bacteria to make fermented milk drinks, especially if the technology of their manufacture involves mixing [2]. Thus, selection of EPS-producing *S. thermophilus* strains of and determining the conditions for their industrial growth is an urgent task of biotechnological developments.

Analysis of recent research and publications

The most common problems that arise when making fermented milk products are their low viscosity and a high level of synaeresis. These problems are often solved by increasing the mass fraction of milk solids or by adding structure stabilisers (modified starch, carrageenan, gelatine, etc.). However, these additives can adversely affect the sensory properties of products. Besides, in most countries of the European Union, it is prohibited to add stabilisers when producing non-fruit-flavoured fermented milk beverages [3].

An alternative method of improving the texture of fermented milk products is the use of LAB strains that produce EPS. It is shown that such cultures play a leading role in the rheological behaviour and formation of the texture of fermented milk by preventing gel destruction, whey separation, and increasing the viscosity. This is mainly due to the formation of bonds among the EPS, the cell surface, and milk proteins [4].

Experts have shown that strains of *S. thermophilus* – EPS producers – form the texture of fermented milk, in particular, by accelerating the coagulation of milk, increasing its viscosity, making a coagulum more plastic and resistant to synaeresis under mechanical impacts (which are inevitable in the reservoir method of manufacturing fermented milk) [5,6].

It has also been reported that there is dependence between the ability to produce EPS and phage resistance. It is shown that phage-resistant strains of *S. thermophilus* are the most productive of capsular polysaccharides and free extracellular slime [7,8].

Some microbial EPS, along with the ability to improve the texture of fermented milk products, also show immunomodulatory, antiulcer, anticancer, and cholesterol-lowering activities, which increases or determines the functional (sometimes therapeutic) properties of finished products [9,10].

The purpose of the work is to select EPS-producing phage-resistant strains of *S. thermophilus* and develop a biotechnology of a bacterial concentrate with their use.

Research **objectives**:

– to investigate the effective viscosity of fermented milk coagula obtained by fermentation using *S. thermophilus* strains;

– to carry out morphological analysis of *S. thermophilus* strains for the ability to form capsules;

– to select EPS-producing phage-resistant *S. thermophilus* strains;

– to establish the conditions of production, storage, and use of the bacterial concentrate of *S. thermophilus.*

Research materials and methods

Object of research: strains of *S. thermophilus* (working numbers 1 to 18) from the collection of the Department of Biotechnology of the Institute of Food Resources, which were maintained in 10% sterile skim milk. The effect of individual substances on the growth of *S. thermophilus* was studied in growth media (GM) of different compositions at 39±1°C for 12 h, inoculum 3%. The components of the GM are given in Table 1.

Table 1 – Composition of the growth media for *S. thermophilus*

Components	Medium											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Lactose [1], %	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	–	2.0	1.0	1.0
Glucose [1], %	–	–	–	–	–	–	–	–	2.0	–	–	–
Sodium citrate, %	–	–	–	0.50		0.50	–	–	–	–	–	–
Sodium acetate,%	–	–	0.50	–	0.50	–	–	–	–	–	–	–
Peptone,%	–	–	–	–	–	–	–	–	–	–	1.0	–
Magnesium sulphate,%	–	–	–	–	–	–	0.05	–	–	–	–	–
Ascorbic acid [1], %	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Yeast autolysate, %	–	2.0	–	–	2.0	2.0	–	–	–	–	–	–
Maize extract, %	–	–	–	–	–	–	–	2.0	–	–	–	–
Reconstituted milk (10%)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mixture of amino acids [2], mg/ml	–	–	–	–	–	–	–	–	–	–	–	0.1
Tapwater, dm^3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[1] sterilised separately at 112°C for 20 minutes;

[2] glutamic acid, methionine, phenylalanine, and leucine in equal proportions

The technological parameters of culturing *S. thermophilus* were practised in the GM developed (which was deacidified every hour with 25% ammonium hydroxide solution to maintain the pH at the level 6.6-7.0 units) at 39±1°C in a bioreactor BIOR 0.1 with the effective space 70 dm^3 at the IFR State Research Enterprise. The protective medium (PM) was prepared from individual sterile components: 10% of sucrose, 5% of sodium citrate, 5% of reconstituted skim milk powder, 10% of inulin. The effectiveness of the PM was evaluated as follows. After 10 h of growth, the biomass (BM) was separated from the culture fluid by centrifugation at 15.000 rpm at 8±2°C, mixed with the PM in the ratios 1:1 and 1:2, and poured into 1 ml bottles. The cells in them were counted before and after freeze-drying. The suspension was frozen in a freezer at minus 60±1°C in 16±2 h, and dried in a freeze-dryer TG 15. The drying started at minus 60±1°C, ended at plus 32±2°C, with the residual pressure not more than 6.65 Pa (0.679kgc/m^2), and lasted 24±2 h, till the residual moisture was not more than 5%.

The effective viscosity was determined using a rotational viscometer RHEOTEST II with a cylinder-cylinder test system (S/S_3) [11]. The capsule-forming ability of the strains was evaluated by immersion microscopy, with the preparations made by the Gins method [12]. The micropreparations were analysed using a light microscope Motic (Fischer Bioblock) with a built-in TopView video camera. The size of the capsules was measured using the software Motic Images 2000 (version 1.3).

The microbiological and sensory characteristics were determined by commonly used methods. The optical density was determined with a photoelectrocolourimeter KFK 3 [12].The storability of the bacterial concentrates (BC) was determined by the change in the number of bacteria in 1 g of a BC, and by the change in its milk coagulation activity after adding 1 dm^3 of 10% pasteurised skim milk [12]. The bacteriophage susceptibility of the strains was determined by the double-layer agar technique, with addition of 10 mM of $CaCl_2$ [13]. The growth parameters of the periodic culture (specific growth rate, μ, h^{-1}, and biomass concentration, X, mg/ml) were calculated according to [14], based on the fact that $(1.00\pm0.02)\times10^{10}$ CFU/ml was 6.1–6.3 mg/ml of dry bacterial residue. The solubility and moisture content of the dry preparation were determined according to [12]. The results were graphically processed using Microsoft Exel 2016. To process the statistical data, analysis of variance was employed [15], using the programmes Statistica 6.0. The research results were evaluated by the significance level P, the experiments were conducted in triplicate.

Results of the research and their discussion

It has been studied how *S. thermophilus* strains (Nos. 1-18) can produce EPS, which, according to [2,6], can improve the rheological properties of fermented dairy products. It has been established that the values of the effective viscosity of fermented milk coagula at the circular velocity 1 m/s (coefficient B) ranged from 7.35±0.22 mPa×s to 171.6±17.8 mPa×s, depending on the strain. Analysing the intergroup variance using the Kruskal–Wallis H test has confirmed statistically significant differences in the effective viscosity of fermented milk coagula formed by the strains under study, H_{fact} 66.650>H_{test} 32.671, $p<0.05$. Therefore, the ability of *S. thermophilus* to produce EPS is a strain-specific feature. It has been found that between such parameters as the size of exopolysaccharide capsules of strains and the effective viscosity of fermented milk products during their fermentation, there were close relationships, and the correlation coefficient was positive: r=0.74 (Fig. 1).

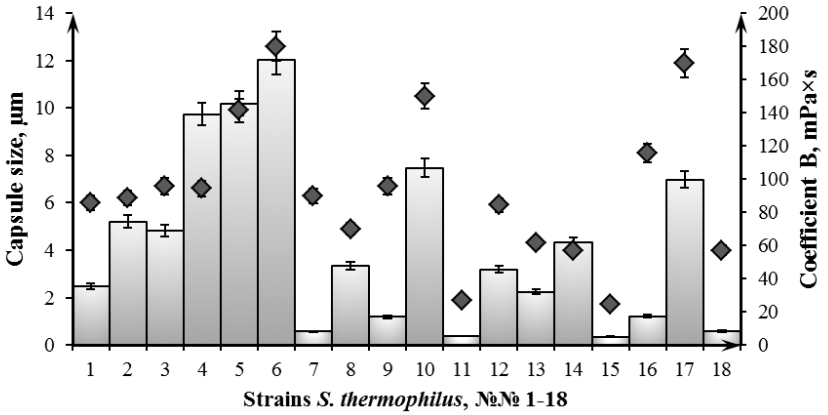

Fig. 1. Change in the viscosity of the products depending on the size of S. thermophilus capsules (strains Nos. 1–18): capsule size – columns; effective viscosity – markers

The actual value of the Student's t-test 4.491 exceeded the critical value of the Student's t-test 2.12, so the dependence of the parameters studied (the size of strain capsules and the effective viscosity of fermented milk products) was statistically significant ($p<0.01$). Phage-resistant strains of *S. thermophilus* Nos. 2-6, 10, 14, and 17, which can produce EPS (free and capsule ones), have been selected as promising for using in biotechnological development of dairy products (Fig. 1).

The strains have been included in the collection of industrial cultures of the IFR (NAAS), with numbers 2178, 2196, 2193, 2192, 2185, 7727, 7728, and 2173, and deposited in the Institute of Microbiology and Virology, NASU. The results are consistent with other researchers' data that the presence of EPS is important in the mechanism of natural phage resistance of LAB: they reduce the sites of adsorption of specific phages on the bacterial cell surface, complicate the progress of phages to a cell through the slime layer, and mask certain receptors [7].

Today, strains that have become phage-resistant due to natural mechanisms, without genetic modification, are widely used in the dairy industry [16].

Testing the technological parameters and modes of production of the BC Iprovit – Streptococcus thermophilus was carried out in order to maintain the preparation's high activity, rapid reactivation, and solubility. It is known that *S. thermophilus* requires the presence of carbon nutrition sources, nitrogen-containing components, vitamins, and trace elements in the GM [17-18]. The growth activity of *S. thermophilus* has been studied in GM of different compositions. The basis of the media was 3% skim milk reconstituted from powder. It was hydrolysed with protosubtilin, with the addition of: 1.0% of lactose and 0.05% of ascorbic acid (No. 1); 2.0% of yeast autolysate (Nos. 2, 5, and 6); 0.5% of sodium acetate (Nos. 3 and 5); 0.5% of sodium citrate (Nos. 4 and 6); 0.05% of magnesium sulphate (No. 7); 2.0% of maize extract (No. 8); 2.0% of glucose (No. 9); 2.0% of lactose (No. 10); 1.0% of peptone (No. 11); and a mixture of amino acids (No. 12). It has been found that increasing the proportion of carbohydrates to 2% in Nos. 9 and 10 led to substrate inhibition of strain growth: the optical density was, respectively, by 0.10–0.15 extinction units lower than in the control medium No. 1, $p\leq0.05$. The addition of sodium acetate (No. 3) and maize extract (No. 8) also inhibited the development of *S. thermophilus*. On the contrary, enriching the media with yeast autolysate (No. 2) and sodium citrate (No. 4) stimulated the growth of LAB. The combination of these components in No. 6 intensified bacterial growth the most: the optical density fluctuated within 0.76-0.92 extinction units depending on the strain. The addition amino acids to No. 12 (methionine, leucine, phenylalanine and glutamic acid, 1:1:1) increased the yield of strains. However, the greatest stimulating effect was that of peptone as an additional source of nitrogen nutrition (Fig.2).

Taking into account the results obtained, the composition of a GM for *S. thermophilus* accumulation has been developed. It is based on reconstituted skimmed milk powder (3.0%) hydrolysed with protosubtilin with the addition of lactose, glucose and peptone (1.0% each), yeast autolysate (2.0%), sodium citrate (1.0%), magnesium sulphate and ascorbic acid (0.1% each), and a mixture of amino acids (0.1 mg/ml). The carbohydrates to soluble nitrogen ratio is C:N=3.5. The study of the accumulation of *S. thermophilus* biomass (BM) in the GM developed has shown that when 7% of inoculums were introduced into the medium for 10 hours, with the pH of the culture fluid maintained at the level 6.6-7.0 units, the BM yield was X=2.62±0.02 mg/ml. This is by 37.1 and 23.7% more than it is when applying, respectively, 3 and 5% of the inoculum. In this variant, the biggest values of the specific growth rate $\mu_{max}=0.89\pm0.03h^{-1}$ in the log phase were recorded (Fig. 3).

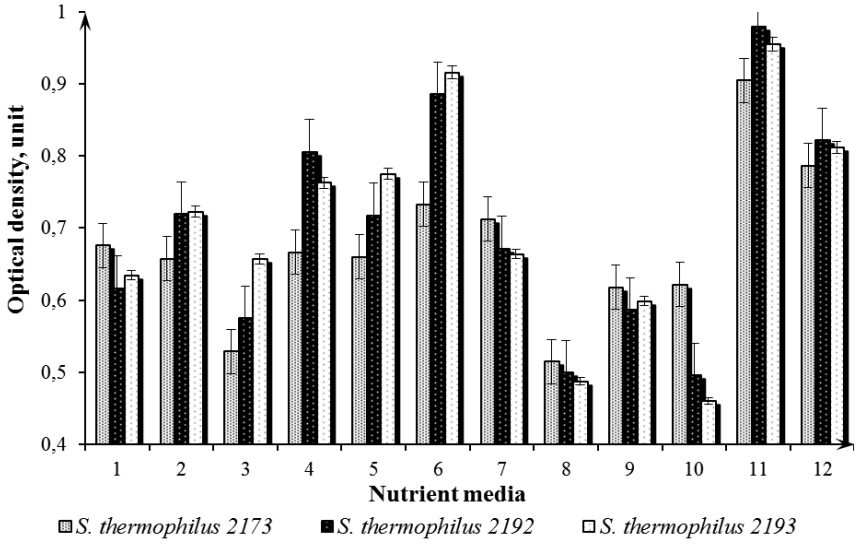

Fig. 2. Growth of *S. thermophilus* in media of different compositions

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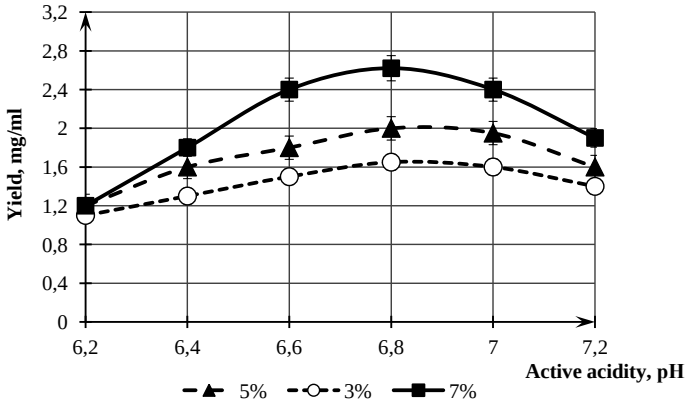

Fig. 3. Accumulation of *S. thermophilus* biomass depending on the amount of inoculum and the acidity of the culture fluid

It has been found that the percentage of preserved *S. thermophilus* cells after such technological operations as freezing and drying is quite high. The variant in which BM was mixed with the PM as 1:2 is better for most strains. Under such conditions, the survival of bacteria reached the level 97–100% (Table 2).

Experimental batches of the BC *Iprovit – Streptococcus thermophilus* have been made using different phage-resistant strains of EPS-producing *S. thermophilus*. The output of dry BC was 7.83±0.02 g from 1 dm^3 of the GM. The well-selected highly active EPS-producing strains in the composition and the carefully selected conditions of BM stabilisation (freezing to minus 60±1°C for 16±2h; use of a special PM, with inulin as a protector) have allowed achieving the high activity and rapid reactivation of concentrates introduced into milk. The BC obtained were characterised by good physicochemical and microbiological parameters, contained 4.4×10^{10} to 1.1×10^{11} viable cells in one gram, were highly soluble (with 0.8±0.06cm^3 of raw sediment), and their moisture content did not exceed 4.7±0.1%.

The duration and conditions of storing the BC have been defined: no more than 18 months at a temperature from minus 18 to minus 20°C; no more than 9 months at a temperature of 4-6°C and a relative humidity of 70-75%. Under such conditions, by the end of storage, the decrease in viable microflora was less than 10% (Table 3).

A method has been developed to modify the rheological properties of dairy products. It involves the technology of dry mixing of basic concentrates to make fermented milk products from with the obtained concentrates *Iprovit-Streptococcus thermophilus* to expand the range of products according to consumer preferences. The results of studying the rheological parameters of products obtained by fermentation with these mixtures are given in Table 4.

Table 2 – Survival of *S. thermophilus* during freeze-drying

Strains, number in the IFR NAAS catalogue	Raw biomass [1], lg CFU/ml		Dry preparation[1], lg CFU/ml		Preservation of cells[1], %	
	BM:PM, 1:1	BM:PM, 1:2	BM:PM, 1:1	BM:PM, 1:2	BM:PM, 1:1	BM:PM, 1:2
2178	8.653	8.477	8.580	8.362	99.16	75.89
2196	8.875	8.748	8.851	8.740	97.26	98.93
2193	8.544	8.230	8.431	8.000	79.23	97.21
2192	8.663	8.079	8.505	8.061	76.17	77.22
2185	8.778	8.342	8.653	8.255	83.93	74.56
7727	8.875	8.748	8.851	8.740	97.26	98.93
7728	8.531	8.000	8.380	7.978	71.56	99.72
2173	8.544	8.255	8.462	8.255	84.93	100.0

[1] arithmetic mean error 1.0%, $p<0.05$

Table 3 – Changes in the microflora of the BC *Iprovit-Streptococcus thermophilus* during storage

Storage time, month	Number of bacteria[1] in g, lg		Milk coagulation activity[1], h	
	minus (18-20)°C	4-6°C	minus (18-20)°C	4-6°C
3	11.38	11.24	5.0	5.0
6	11.30	10.58	5.0	5.5
12	10.89	9.21	5.5	6.5
18	10.56	8.59	6.0	7.5

[1] arithmetic mean error 1.0%, $p<0.05$

Table 4 – Changes in the viscosity of fermented dairy products depending on the mixing recipe

Basic concentrate (BC)	*Iprovit – Streptococcus thermophilus* (TC)	Mix, BC:TC	Increase in the effective viscosity[1)], %
BC for fermented milk	2178	1:0.05	26.1
		1:0.1	50.2[2)]
BC SSC for sour cream	2193	1:0.1	30.1
		1:0.5	54.3[2)]
BC KM for kefir	2196	1:0.05	15.1
		1:0.1	41.4[2)]
BC for bifidobacteria-based yoghurt	7727	1:0.05	13.3
		1:0.1	39.2[2)]
BC *Immunolactovit* for milk beverages	2192	1:0.05	17.9
		1:0.1	51.5[2)]
BC *Bifidolact* for milk beverages	2173	1:0.1	19.6
		1:0.2	48.2[2)]

[1)] arithmetic mean error 1.0%, $p \leq 0.05$; [2)] the difference is significant at $p \leq 0.05$

The technology is applicable quite universally. It allows changing the texture of products easily: the effective viscosity of the fermented products increased by 13.3–54.3% (depending on the formulation). At the same time, the products retained their original taste and aroma composition, since the dose of EPS producers added was very small (due to the activity of the preparations obtained), and ranged from 45 to 182g per 1kg of the mixture.

Approbation of results

The conditions established for the manufacture of the BC *Iprovit – Streptococcus thermophilus* are included as normative in the Technological Instructions for the Production of Fermenting Cultures *IPROVIT* (TU U 15.5-00419880-100:2010 "Dry and liquid fermentation cultures. Specifications"). The novelty of the technological solutions is confirmed by Copyright Registration Certificate No. 51033. The BC *Iprovit - Streptococcus thermophilus* was tested and implemented in a production environment at the IFR State Research Enterprise, NAAS of Ukraine.

Conclusion

This research has resulted in establishing the differences in the production of exopolysaccharides by *S. thermophilus* strains. It has been shown that between the size of exopolysaccharide capsules of *S. thermophilus* strains and the effective viscosity of dairy products during their fermentation, there are correlations r=0.74, t-Student=4.491, p<0.01. Therefore, it is advisable to use morphological analysis of capsules of LAB strains as a criterion for selecting strains to be included in the BC composition. After screening, phage-resistant strains of EPS-producing *S. thermophilus* have been selected as promising for use in biotechnological developments. Two strains have been protected by Patents of Ukraine 92287 and 91441. The conditions of growing EPS-producing *S. thermophilus* industrially have been determined. In particular, the optimum composition of the growth medium has been developed, with the carbohydrates to soluble nitrogen ratio C:N=3.5 as the most practical. It has been determined that the development was the most active with 7% of inoculum introduced and the pH of the culture fluid maintained at the level 6.8±0.2 units. Due to using a protective medium with inulin (10%) as a protector to be mixed with the biomass separated from liquid, freezing the suspension at minus 60±1°C for 16±2h, and drying it for 24±2h till the residual moisture did not exceed 5%, it has become possible to achieve high cell survival activity 97-100%, and to make the bacterial concentrate well-soluble and rapidly reactivated when introduced into milk. Concentrates of different *S. thermophilus* strains contained not fewer than 4.4×10^{10} CFU/g, the output was 7.83±0.02 g from 1 dm^3 of the medium. The concentrates obtained have been used in the technology of mixing with other concentrates to manufacture fermented dairy products with the viscosity increased by 13.3–54.3%, depending on the mixing recipe. The taste of the products obtained was pleasant, reminding of sour-milk, retaining the individual features of the products characteristic of this or that type of concentrate.

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РОЗРОБКА БІОТЕХНОЛОГІЇ *STREPTOCOCCUS THERMOPHILUS*-ПРОДУЦЕНТІВ ЕКЗОПОЛІСАХАРИДІВ

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Анотація. Проведено скринінг та відібрано фагостійкі штами *Streptococcus thermophilus* – продуценти екзополісахаридів, перспективні до застосування для природного, дієвого та безпечного способу формування текстури ферментованого молока, покращення реологічних властивостей кисломолочних продуктів. Запропоновано як експрес-метод для оцінки реологічних властивостей використовувати аналіз капсулоутворювальної здатності штамів. Розроблено технологію бактеріального концентрату «Іпровіт-*Streptococcus thermophilus*» з фагостійкими продуцентами екзополісахаридів. За рахунок встановлених технологічних режимів: нарощування у живильному середовищі зі співвідношенням вуглеводів до розчинного азоту, як C:N=3.5; стабілізація біомаси (захисне середовище з інуліном 10%, заморожування за мінус (60±1)°С впродовж (16±2) год, сушіння впродовж (24±2) год до плюс (32±1)°С, волога у препараті не більше 5%) одержали (7.83±0.02) г бактеріального концентрату з 1 дм^3 середовища. Сухий бактеріальний концентрат містив (4.4×10^{10}-1.1×10^{11}) КУО/г активної мікробіоти залежно від штаму *Streptococcus thermophilus*. Розроблено технологічний прийом покращення реологічних властивостей кисломолочних продуктів змішуванням бактеріальних препаратів для виробництва різноманітної кисломолочної продукції з концентратом штаму *Streptococcus thermophilus*. Підібрано оптимальні рецептури сумішей, що дало можливість коригування реологічних характеристик ферментованих молочних продуктів, не застосовуючи інших згущувачів. Ефективну в'язкість отриманих продуктів збільшили на (13.3-54.3)% залежно від рецептури суміші. Біотехнологію бактеріального концентрату «Іпровіт-*Streptococcus thermophilus*» впроваджено на Державному дослідному підприємстві Інституту продовольчих ресурсів НААН України. Виробництво концентрату регламентовано у розроблених та затверджених нормативних документах: ТУ У 15.5-00419880-100:2010 «Культури заквашувальні сухі та рідкі. Технічні умови», Технологічній інструкції з виробництва заквашувальних культур «ІПРОВІТ» до ТУ У 15.5-00419880-100:2010 «Культури заквашувальні сухі та рідкі. Технічні умови», оригінальність та новизну технологічних рішень підтверджено Свідоцтвом про реєстрацію авторського права № 51033.

Ключові слова: *Streptococcus thermophilus,* фагостійкі штами, продуценти, екзополісахариди, капсули,

ефективна в'язкість, біотехнологія.

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