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LACTIC ACID BACTERIA HYDROLYSATES AND THEIR EFFECT ON SKIN HYDRATION

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Abstract. Recently, interest in lactobacilli lysates is growing, and the possibilities of their use cover more and more areas of human life – medicine, immunoprophylaxis, cosmetology, food industry. The article presents a method of obtaining bacterial lysates of lactic acid bacteria, Lactobacillus genus. As a destructive agent used dry lytic enzyme preparation cytal-Rk G-10X, obtained under conditions of experimental fermentation from the culture fluid *Streptomyces albus* UN44. The complex lytic preparation cytal-Rk contains a group of enzymes glycosidases and peptidases, the joint action of which leads to the degradation of the cell wall of a wide range of bacteria. The evaluation of the effectiveness of the use of Cytal-Rk for the degradation of six strains of lactobacilli is presented. It is shown that the enzyme is an effective destructive agent under optimal conditions. The degree of cell degradation depended on their species, hydrolysis conditions and physical condition. Optimal conditions for obtaining hydrolysates based on native and lyophilized cells of *L. delbrueckii subsp. bulgaricus* LB86 was develop. When loading the microbial mass in the reaction medium – 1×10^9 CFU/ml (for native cells) and 1×10^{10} CFU/ml (for lyophilized cells) cell destruction reached almost 80% and 90%, respectively. Based on native *Lactobacillus delbrueckii subsp. bulgaricus* LB86 lysates were obtained and their comparative chemical analysis was performed. Sublimated cells were shown to be more sensitive to the enzyme complex and to contain more proteins and reducing sugars. Native cell lysates were more enriched in nucleic acids. Lysate of lactic acid bacteria from native cells was studied to moisturize the skin of the hands of young people aged 18–20 years. When using lysate in the cream base, it significantly increased the level of hydration of the dermis of the hands compared to the control cream base. This allows us to consider the lysate of *Lactobacillus delbrueckii subsp. bulgaricus* LB86 as a promising ingredient for creating cosmetics with moisturizing action.

Key words: lysates, *Lactobacillus*, enzymatic disintegration, cosmetic, skin hydration

Introduction. Formulation of the problem

The great interest in bacterial lysates is due to the content of a significant pool of biologically active substances, such as nucleic acids, free fatty acids, organic acids, amino acids, structural and functional proteins, sugars, cell wall components, fragments of cytoplasmic membranes and more. At present, various methods are used to obtain bacterial lysates – physical, chemical and enzymatic, but some of them have a number of disadvantages. Thus, physical methods often lead to overheating of cellular structures, which promotes denaturation and aggregation of proteins. The use of aggressive substances in chemical lysis can pose a significant risk to human health. The most gentle method of bacterial disintegration is the hydrolysis of cells by lytic enzymes, which destroy the polymer structure of the peptidoglycan of cell walls. Lysozyme is most often used for these purposes. However, lysozyme has a certain spectrum of antibacterial action and some bacteria are insensitive to it. In addition, lysozyme is obtained from chicken egg protein and its industrial use is not always justified. Therefore, the search for new cheap but effective enzyme preparations obtained by microbial synthesis is of interest for use in biotechnological industries, in particular, in the production of microbial lysates.

Analysis of recent research and publications

Lactobacilli are important representatives of the resident microflora of humans, which has a high biotherapeutic potential [1,2]. They have antagonism to a relatively wide range of conditionally pathogenic microorganisms [3], participate in lactose metabolism [4], affect cholesterol and mineral metabolism [5]. In pathological conditions, lactobacilli maintain the balance of steroid hormones [6], exhibit antioxidant [7,8], antimutagenic [9] and anticarcinogenic properties [10]. Probiotics also actively affect the skin microbiome [11].

It is known that lysates derived from bacterial cells contain a pool of cellular metabolic products and fragments of cellular structures. Their main advantage is immunostimulatory activity due to activation of cellular and humoral immunity without the risk of provoking inflammatory reactions [12]. In recent years, lysos of lactobacilli have been actively used in dermatocosmetology. This is due to the fact that they are able to increase the protective function of the skin and activate reparative processes in it [13,14]. Under the action of lysates, there is a marked decrease in the clinical sensitivity of the skin by reducing the reactivity and accessibility of neurons to the external stimulus [15]. There is strong evidence that lysates can be safe and effective immunobiotics for the treatment and prevention of eczema and other atopy [16].

Recently, the problem of dry skin or xeroderma has become widespread among the population [17]. This is especially true during a pandemic, when alcohol-based products are used for disinfection. They not only dry out the skin, but also lead to disruption of the skin microbiome, damage to the epidermal barrier, atopy, inflammation, etc. The use of bacterial lysates as ingredients in cosmetic products that reduce the sensitivity of reactive skin, protect the skin barrier and increase skin moisture may be especially relevant in modern conditions. However, the evidence base for the action of lysates in cosmetics is still insufficient [18] and requires additional research..

At the present time, obtaining bacterial lysates is carried out by disintegration of their cell walls by various methods – physical, chemical or biological [19]. But there is no systematic approach in the scientific literature regarding the effectiveness of the methods of obtaining them. This is due to the fact that the degree of disintegration of cells of microorganisms depends, first of all, on the features of the structure of their cell walls [20]. This fact does not allow to choose the universal method of obtaining bacterial lysates, but requires an individual approach to each type of microorganisms separately.

The most popular method of destruction of bacterial cells is hydrolysis by proteolytic enzymes that are able to break down glycosidic and peptide bonds in their cell wall. Lysozyme is most often used for these purposes. There are data on the use of other proteolytic enzymes [21–23]. Recently, the use of a combination of several enzymes or a combination of several methods has been practiced to obtain lysates [21,24]. Scientific studies have shown that probiotics and their lysates have a large range of biotherapeutic properties. However, the functional activity of lysates depends on the nature of the lysed bacteria and the method of their hydrolysis. This necessitates the search for new hydrolyzing enzymes and bioactive strains of bacteria for the development of multifunctional ferment lysates on their basis.

The **purpose** of the work – obtaining and characterization of fermentolysates of lactic acid bacteria Lactobacillus genus for the creation of cosmetics

The main **objectives** of the study:

– to determine the optimal conditions for hydrolysis of lactobacilli cells when used as a lysing agent of the enzyme preparation Cytal-Rk;

– obtain a lysate of lactobacilli and investigate its chemical composition;

– determine the effect of lysate on skin hydration.

Research materials and methods

The subject of the study were strains of *Lactobacillus* lactic acid bacteria, the list of which is shown in Table 1.

Lyophilized *L. delbrueckii subsp bulgaricus* LB86 cells were used in the studies. To obtain fermentolysates dry lytic enzyme preparation Cytal-Rk G-10X (activity – 7000 U/g) was used, obtained from the culture fluid of *Streptomyces albus* UN44 under conditions of research fermentation on Department of Industrial Biotechnology Igor Sikorsky Kyiv Polytechnic Institute. The reference preparation is lysozyme from egg white (≥ 30,000 FIP-U/mg), manufactured by Merck KGaA, Germany.

Table 1 – **Test strains of *Lactobacillus* sp.**

The name of the strain	Origin
L. rhamnosus LB3 (IMB B-7038)	Non-commercial lactic acid products
L. murinus DSM 20452	German Collection DSMZ Biological and enzyme
L. delbrueckii subsp. *bulgaricus* LB86 (ВКПМ B-5788)	Biological and enzyme preparations plant ENZIM
L. acidophilus (C)	Non-commercial lactic acid products
L. rhamnosus (C)	Institute Rossell INC, Canada
L. delbrueckii subsp. *bulgaricus* LB51	DK Zabolotniy Institute of Microbiology and Virology, NAS of Ukraine

The reaction mixture included a suspension of lactic acid bacteria cells (10^9 cells/ml) and Cytal-Rk with a concentration of 100 mg/ml. The concentration of the enzyme was chosen according to our own research. Lysozyme with a concentration of 100 μg / ml was used in control samples [25]. Cell lysis was performed under the temperature optimum Cytal-Rk – 55°C [23] and the temperature range of lysozyme [26].

The lytic sensitivity of LAB strains was determined by the turbidimetric method on PEC-3 (10 mm cuvette, 540 nm wavelength) according to the change of the optical density of the primary and final samples and was calculated in % according to the formula (1):

$$OD,\% = \frac{OD_{init}^{540} - OD_{fin}^{540}}{OD_{init}^{540}} \cdot 100\% \qquad (1),$$

where $OD,\%$ – cell lysis, %;

OD_{init}^{540} – the optical density of the cell suspension before incubation;

OD_{fin}^{540} – the optical density of the cell suspension after incubation.

Heat treatment of the lysate was performed by autoclaving in the mode: 0.8 atm, 30 min. The protein content in the lysates was investigated by colorimetric method at a wavelength of 750 nm [27]. The content and composition of nucleic acids were determined by spectrophotometric method. The change in the difference between the optical density of the hydrolysates was measured at 270 nm (maximum absorption of nucleic acids) and 290 nm (maximum absorption of impurities) [28]. Determination of the concentration of reducing sugars was determined by a standardized method: DSTU 5059: 2008 Confectionery. Methods for determining sugars. The activity of acid formation was determined by a standardized method: DSTU8550:2015 Milk and dairy products. Ph measurement by potentiometric method.

The lysate was used as part of a cosmetic cream base prepared from apricot kernel oil (Aroma-Zone) at a concentration of 20%, with the addition of emulsifier Olive 1000 (Aroma-Zone) at a concentration of 6.0%, preservative Cosgard (Aroma-Zone) 0.6% and distilled water. The concentration of lysate (by protein) in the cream was 38.64 mg/ml. Cream base without lysate (K1) was used as a comparison cream. The cream base was applied to the skin of the left wrist and the cream with lysate on the skin of the right wrist twice a day – morning and evening. The study involved 9 volunteers aged 18–20 years (6 girls and 3 boys) with irritated, dry skin (less than 30 CM units). Measurement of skin moisture was performed before applying the cream (control value (K_0) after 2 hours, 48 hours and after 7 days. The level of hydration of the skin surface was determined by measuring the electrical capacity of the stratum corneum using a corneometer (Courage and Khazaka CM202PC, Germany). The results were expressed in conventional units (CM units) [29].To compare the hydration estimates of the cream base and the lysate cream, the mean difference points for the treated and untreated areas of the skin at each time point were calculated.

Statistical data processing. All digital data obtained was processed using Excel from Microsoft Office-2010. The numerical data are presented as arithmetic mean and standard error (M ± m).

Results of the research and their discussion

The complex lytic preparation Cytal-Rk contains a group of enzymes glycosidases and peptidases, the joint action of which leads to degradation of the cell wall of bacteria [30]. To evaluate the efficacy of Cytal-Pk, screening of its activity against 6 strains of lactic acid bacteria (LAB), which were in the stationary phase of growth, was conducted. Lysozyme was used as a control (Fig. 1).

According to Figure 1, Enzymes also had a different spectrum of action. Cytal-Rk was effective against 5 cultures: *L. rhamnosus* LB3, *L. murinus*, *L. acidophilus* (C), *L. delbrueckii* subsp. *lactis* LE, *L. delbrueckii* subsp. *bulgaricus* LB86. Lysozyme had advantages only in relation to the strain: *L. rhamnosu*s (C)The sensitivity of LAB to the effect of Cytal-Pk varied within 30.0–39.7%. Sensitivity to lysozyme ranged from 3.2–48.6%. Weak sensitivity of lactobacilli to lysozyme has been reported in a number of studies. It has been suggested that low sensitivity to the enzyme of some strains of lactobacilli may be due to the presence on the surface of their cell walls S-layer that protects cells from damage [31,32].

Selective action of enzymes probably depended not only on the peculiarities of the structure of the enzymes, but also on the chemical nature of the cell walls of lactobacilli The efficiency of hydrolysis is largely determined by the conditions in which it is carried out. Table 2 shows the effect of pH and duration of hydrolysis on the activity of Cytal-Pk against *L. delbrueckii subsp. bulgaricus* LB86, which was selected to create a lysate based on its probiotic properties [33].

The activity of the enzyme preparation was manifested only at acidic and slightly acidic pH values of 5.8–6.5. At pH values above 7.0, cell lysis did not occur. Taking into account the optimal pH value, the sensitivity of the cells to the action of the enzyme was studied, depending on the age of the culture and the time of hydrolysis. The results are presented in Table 3.

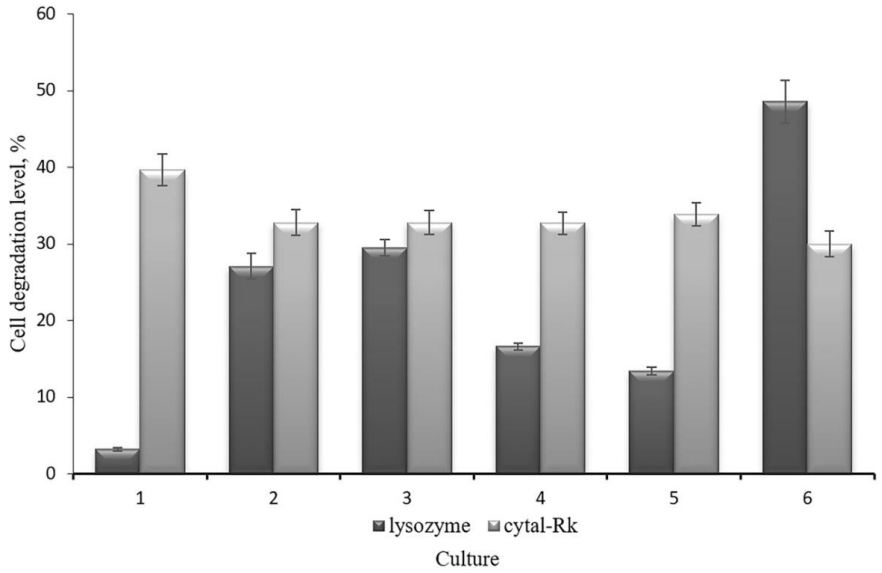

Fig. 1. The lytic activity of cytal-Rk and lysozyme against *Lactobacillus* bacteria (Age of culture – 24 hours, hydrolysis time – 60 minutes, t – 55°C)

1 – L. rhamnosus LB3 , 2 – L. murinus, 3 – L. delbrueckii subsp. bulgaricus LB86, , 4 – L. acidophilus (C), 5– L. delbrueckii subsp. lactis LE, 6 – L. rhamnosus (C)

Table 2 – Effect of pH on duration of hydrolysis on the activity of enzymatic lysis of *L. delbrueckii* subsp. *bulgaricus* LB86 (n = 3, p≤0.95)

pH	Cell lysis activity, %		
	60 min	120 min	180 min
6.0	29.1±0.54	35.3±0.54	39.4±0.54
7.0	0	0	0
8.0	0	0	0
DH_2O (pH 6.5)	30.6±0.43	34.5±0.43	40.1±0.43
DH_2O (pH 5.8.)	31.8±0.49	32.1± 0.49	32.8±0.49

Table 3 – Sensitivity of *L. delbrueckii* subsp. *bulgaricus* LB86 to Cytal-Rk from the age of the culture and the time of hydrolysis (n = 3, p≤0.95)

Time of cultivation of culture, h	Cell lysis activity, (%) at the time, t (min)		
	60 min	**120 min**	**180 min**
Control (primary conditions)	31.8±0.28	32.1±0.36	32.8±0.40
12	52.9±0.31	62.5±0.34	63.4±0.40
14	58.5±0.34	67.0±0.37	75.2±0.38
16	58.8±0.47	66.2±0.48	79.0±0.48
24	33.1±0.46	50.6±0.44	57.9±0.45

The maximum sensitivity to the enzyme preparation of the cells was shown at 14–16 hours of growth and the hydrolysis time of 180 min. Thus, to obtain fermentolysates in the future, the optimal conditions were observed: the age of culture 14–16 h, pH – 6.5, temperature – 55^0C, hydrolysis time – 180 min.

Figure 2 shows the lysis of the studied culture in dynamics under primary conditions (culture age – 24 h, pH – 5.8, hydrolysis time – 60 min) and optimized.

Conducting lysis in the developed conditions allowed to increase the hydrolysis of cells compared with the initial in 2.2 times at 60 min of lysis, 2.1 times at 120 min and 2.4 times at 180 min. Thus, it has been shown that the enzyme preparation Cytal-Rk, under optimal conditions, is an effective disintegrant that allows to obtain hydrolysates with more than 70% efficiency.

To obtain concentrated lysates, the hydrolysis of the dried biomass of the strain *L. delbrueckii* subsp.

bulgaricus LB86 was conducted. The sensitivity of dry biomass cells was shown to be quite high and was 86,9±1,4% at 180 min of lysis (Table 4).

The chemical composition of the native culture lysate and the lysate obtained on the basis of dry biomass were evaluated before and after its heat treatment. Heat treatment of lysate was carried out in order to increase the shelf life. Data on protein content, nucleic acids, reducing sugars and acidity of the obtained lysates is presented in Table 4.

It was determined that lysates have pH values in the range of 5.37±0.10–6.32±0.11. This created favorable conditions for their use in the composition of cosmetics, because the value of the acidity of skin care products, depending on the purpose of their use will have a pH range of 4–9 [34]. The presence of proteins, reducing acids, and nucleic acids was determined in living culture lysates. Dry biomass lysates contained a much higher proportion of proteins and reducing acids. The increase in these substances in dry culture hydrolysates is associated with the majority of hydrolyzed cells. But the content of nucleic acids in them sharply decreased up to their full absence in sterilized lysates.

Since nucleic acids have great potential for use in cosmetic and cosmeceutical preparations [35], lysates obtained from native culture *L. delbrueckii subsp. bulgaricus LB86*. were used for further studies. Lysate as a part of the cosmetic preparation was tested to moisturize the skin of the hands. The results are presented in Fig. 3.

The results show us that twice-daily application of the cream with lysate has led to a significant increase in skin moisture over time. As follows, 2 hours after application of the cream base with lysate (K) on a skin the increase in moisture content was 1.7 times higher than when applying the cream-base (K1).

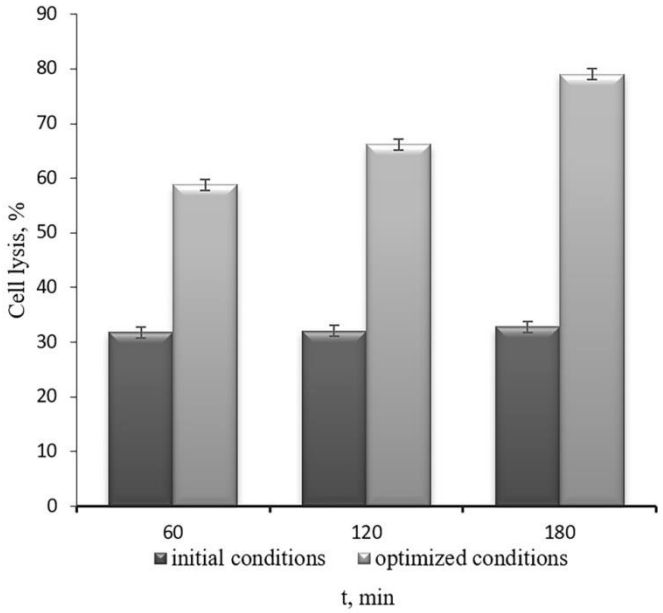

Fig. 2. Enzymatic lysis of *L. delbrueckii* subsp. *bulgaricus* LB86 cells by the Cytal-Rk preparation under different conditions (Load of microbial mass in the reaction mixture – 1×10^9 CFU/ml)

Table 4 – General chemical characteristics of hydrolysates

Lysates	Cells hydrolysis, %	pH	Total acidity, °T	Protein, mg/ml	Reducing sugars, µg/ml	DNA µg/ml	RNA. µg/ml
Lysate from native biomass, 10^9 cells/ml	79.60± 0.37	6.32± 0.11	30.0± 2.0	38.64± 3.6	134.0± 6,0	58.2± 1.49	60.5 ±1.55
Lysate from dry biomass, $1,5\cdot10^{10}$ cells/ml	86.9± 1.4	6.19± 0.08	32.0± 3.0	148,2± 9.0	656.0± 8.5	0.4784± 0.33	0.4974 ±0.315
Sterilized lysate from dry biomass, $1,5\cdot10^{10}$ cells/ml	86.9± 1.4	5.73± 0.10	31.0± 2.1	168.0± 6.0	616.0± 7.8	0	0

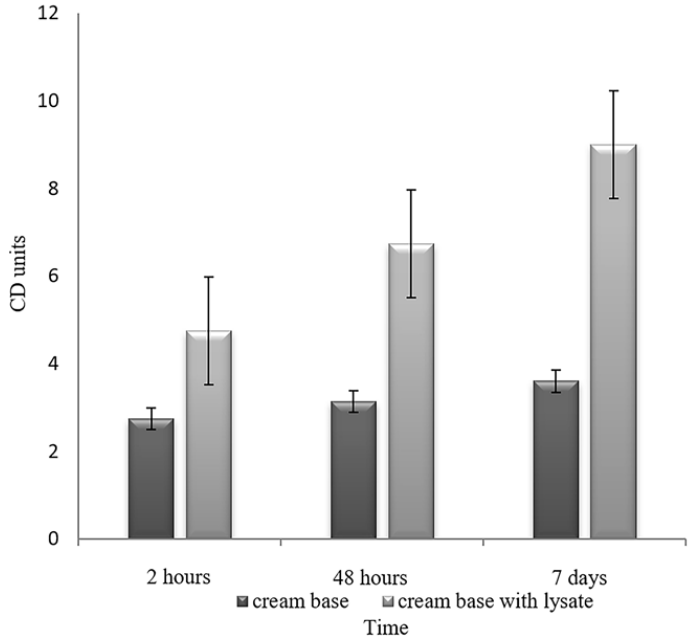

Fig. 3. The subtracting average of hands skin hydration estimates in the dynamics

After 48 hours the difference in moisture content of the skin in the area of application of the cream base with lysate (K) was 2.1 times higher, and after 7 days – 2.5 times higher than in the area of application of the cream base (K1). The cream base also helped to increase skin moisture by 2.6–3.7 CM units from the initial value (K_0), depending on the duration of its use. This is obviously due to the presence of apricot kernel oil, which plays an important role in retaining moisture and restoring the protective layer of the skin due to the high content of linoleic acid. We do not exclude that lysates, which contain a pool of fragments of cellular structuresmay improve the regulation of skin water metabolism by stimulating the synthesis or activity of cellular aquaporins [36] or to affect the content of components of the natural moisturizing factor of the skin [15]. The effect of lysates on skin moisture may be associated with the restoration of skin barrier function by increasing the expression of skin barrier proteins – loricrin and filaggrin [37]. However, the mechanisms of action of lactobacilli lysates and its effect on the skin condition need further study.

Thus, the lysate of lactobacilli *L. delbrueckii subsp. bulgaricus* LB86 in combination with apricot oil can be a promising biologically active substance for the creation of cosmetics with a moisturizing effect. The obtained results are the basis for further research to study the mechanisms of biotherapeutic action of lysates on the skin in its various physiological states.

Conclusion

A method of producing hydrolysates based on native cells of *L. delbrueckii* subsp. *bulgaricus* LB86 was created. With the load of the microbial mass in the reaction suspension $1 \cdot 10^9$ cells/ml, the concentration of the enzyme – 100 mg/ml, the age of culture 14–16 h, the temperature 55°C, pH – 6.5 and the duration of the hydrolysis of 180 min, cell destruction reached more than 70%.

A comparative chemical analysis of lysates obtained from cells that were in different physiological states was performed. Sublimated cells have been shown to be more sensitive to the enzyme complex and contain significantly more protein and reducing sugars. But lysates from native cells were more enriched in nucleic acids.

It has been shown that lysates from native cultures are an effective moisturizing ingredient for hand skin cosmetics. The area of cream application which contained lysate after 7 days showed the difference in skin moisture. It is proved that the moisture level was 2.5 times higher than the control value. The data obtained allows us to broaden our understanding of the functional activity of lactobacillus lysates and to determine the prospects of their use for cosmetic products with targeted action.

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ГІДРОЛІЗАТИ ЛАКТОБАКТЕРІЙ ТА ЇХНІЙ ВПЛИВ НА ВОЛОГІСТЬ ШКІРИ

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Анотація. Останнім часом інтерес до лізатів лактобактерій зростає, а можливості їхнього використання охоплюють все більше сфер життя людини – медицину, імунопрофілактику, косметологію, харчову промисловість. У статті наведено спосіб отримання бактеріальних лізатів молочнокислих бактерій р. *Lactobacillus*. Як руйнівний агент використано сухий літичний ферментний препарат цитал-Рк Г-10Х, що отримано в умовах дослідної ферментації з культуральної рідини *Streptomyces albus* UN44. Комплексний літичний препарат цитал-Рк містить групу ферментів глікозидаз та пептидаз, спільна дія яких приводить до деградації клітинної стінки широкого кола бактерій. Представлено оцінку ефективності використання цитал-Рк для деградації шести штамів лактобактерій. Показано, що фермент є ефективним руйнівним засобом за оптимальних умов. Ступінь деградації клітин залежала від їх виду, умов гідролізу та фізичного стану. Розроблено оптимальні умови отримання гідролізатів на основі нативних та ліофільно висушених клітин *L. delbrueckii subsp. bulgaricus* LB86. При навантаженні мікробної маси в реакційному середовищі – $1 \cdot 10^9$ КУО/мл (для нативних клітин) та $1 \cdot 10^{10}$ КУО/мл (для ліофільно висушених клітин) руйнування клітин досягало майже 80% та 90% відповідно. Отримано лізати на основі нативних клітин *Lactobacillus delbrueckii subsp. bulgaricus* LB86 та проведено їхній порівняльний хімічний аналіз. Показано, що сублімовані клітини, які були більш чутливими до дії ферментного комплексу містили більше білків та редукуючих цукрів. Лізати з нативних клітин були більш збагачені нуклеїновими кислотами. Лізат молочнокислих бактерій з нативних клітин було досліджено на зволоження шкіри рук молодих людей віком 18–20 років. При використанні лізату у складі кремової основи він сприяв суттєвому збільшенню рівня гідратації дерми рук у порівнянні з контрольною кремовою основою. Це дозволяє розглядати лізат *Lactobacillus delbrueckii subsp.bulgaricus* LB86 як перспективний інгредієнт для створення косметичних засобів зі зволожувальною дією.

Ключові слова: лізати, р. *Lactobacillus*, ферментативна дезінтеграція, косметика, зволоження шкіри.