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RATIONAL MODES DEFINITION OF THE OBTAINING OF LOW MOLECULAR WEIGHT PEPTIDES FROM THE *Lactobacillus delbrueckii subsp. bulgaricus* PEPTIDOGLYCANS

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Abstract. The problem of the population immune status reducing should be corrected through nutritional support. Components of the peptidoglycans of probiotic bacteria cell walls (low molecular weight muropeptides) are promising components of functional food ingredients and dietary supplements that have powerful immunotropic activity. It is proposed to obtain target peptides by combining autolysis and enzymatic hydrolysis of biomass *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* peptidoglycans. The effect of the degrading effect of autolysis on biomass was studied in the temperature range of 50–70°C, incubation under these regimes was carried out after 8 hours from the start of cultivation. It is established that the most significant autolytic changes occur when incubating biomass after 8 hours of cultivation at 50°C for 4 h, as evidenced by a sharp increase in the content of amino acids in the culture medium (4.2 mg/cm^3). For deeper hydrolysis of the peptidoglycans of the biomass autolysate, various variations of enzymatic treatment involving lysozyme, papain and a combination thereof were used. To determine the rational conditions of enzymatic hydrolysis, the methods of mathematical planning of multifactorial experiments were used, in which the input factors were: C_L – lysozyme concentration, mg/cm^3; C_P, the concentration of papain, mg/cm^3; τ – process duration, h. The output parameter of mathematical modeling is the concentration of low molecular weight peptides in the hydrolysate (C_{LMWP}, mg/cm^3). A series of experiments were conducted to investigate the features of enzymatic hydrolysis of biomass in order to determine the rational limits of input factors. It has been found that the maximum concentration of low molecular weight peptides C_{LMWP} in hydrolysate occurs in certain ranges of enzyme concentrations: lysozyme C_L= 5–10 mg/cm^3, papain C_P= 10–20 mg/cm^3. By the least squares method and the sequential regression analysis implemented in PLAN program, the regression equations for natural variables that adequately describe the dependence of the output factor on the inputs by the Fisher criterion have been obtained. The rational parameters of enzymatic hydrolysis by lysozyme (C_L= 10 mg/cm^3, τ = 17.63 h, C_{LMWP}=1.48 mg/cm^3), papain (C_P= 11.31 mg/cm^3, τ = 13.72 h, C_{LMWP}= 4.10 mg/cm^3) and enzymes combination (C_L= 5.00 mg/cm^3, C_P= 11.67 mg/cm^3, τ = 13.21 h, C_{LMWP}= 6.72 mg/cm^3) were determined. The content of the target muropeptides is 4.35 mg/cm^3.

Key words: low molecular weight peptides, peptidoglycan, probiotic bacteria, autolysis, enzymatic hydrolysis, lysozyme, papain, mathematical modeling.

Introduction. Formulation of the problem

According to the reports of the Ukrainian Institute for Strategic Studies of the Ministry of Health of Ukraine, the World Health Organization, an increasing number of people suffer from diseases associated with immune system dysfunction. Anthropogenic load, social factors [1-3] provoke a decrease in the immune status of the average person, this effect is very amplified by the defeat of cancer, or infection with immunodeficiency virus, or antibiotic-resistant pathogens [4].

Creating and implementing effective preventative measures is necessary to overcome this global problem. Ithe nutritional support of the population is mportant, namely, the introduction functional foods into the diet, containing immunotropic ingredients that

can help to increase the body's resistance to various diseases.

Analysis of recent research and publications

Promising immunological components of functional food ingredients and dietary supplements are low molecular weight muropeptides, which are degradation products of bacterial cell wall peptidoglycans. It is known that peptidoglycan fragments to be able to penetrate the intestinal barrier [5], stimulating evolutionarily entrenched mechanisms of the immune response. The cleaved peptidoglycan is transported inside the cells and recognized by cytoplasmic receptors (Nod 1 and Nod 2) [6-8]. Once activated, these molecules trigger intracellular signals that lead to the activation of transcriptional responses, culminating in the expression of inflammatory genes [8,9].

Destruction of peptidoglycans of microorganisms cell walls is carried out using physical, chemical, biochemical or combined methods of exposure. Most of the known methods for producing muropeptides [10-11] are quite complex to perform, especially on an industrial scale. They are multi-stage, with the using specific reagents.

Typically, a combination of physical and enzymatic methods of exposure is used to obtain components of peptidoglycans of regular structure [11], moreover, from the physical methods of bacterial cell disintegration, mainly ultrasonic treatment is used [12]. For enzymatic catalysis of the specific peptidoglycan bonds, the proteases, muramidases, or a combination thereof are typically used [13,14]. The proteases most commonly used are pepsin, trypsin, chymotrypsin or the complex enzyme preparation pancreatin. As muramidases is used lysozyme, or mutanolysin, which is a metabolite of *Streptomyces globisporus* ATCC 21553.

To obtain immunological food ingredients based on bacterial peptidoglycans, it is advisable to use probiotic cultures of microorganisms. Probiotic bacteria contain up to 70% of peptidoglycans and are absolutely safe [15,16].

Typically, known methods of bacterial cell destruction are carried out in order to investigate the architecture of their cell walls and to find effective means for the destruction of pathogens. Little attention has been paid to the study of the degradation features of probiotic bacteria for the production of low molecular weight hydrolysis products of peptidoglycans as promising immunological food ingredients.

Given into the account the specificity of the probiotic bacteria peptidoglycans structure [17], it is advisable to use proteases having a wide range of substrate specificity for their destruction. There are no data in the literature on the efficacy of using the protease papain to produce low molecular weight degradation products of bacterial peptidoglycans.

Papain is protease that has lower substrate specificity than other proteases, the papain exhibits maximum activity in the range of pH 5–7.5 units [18]. Due to this, it is possible to combine this enzyme with muramidase lysozyme, that has maximum enzymatic activity in the range of pH 4.5–5.5 units. This can increase the depth and hydrolysis efficiency of specific peptide and glycosidic bonds of peptidoglycan. The ability of probiotic bacteria to synthesize a number of autolysins enzymes capable to cleave specific peptidoglycan bonds [19-21] should also be taken into account.

Combining these methods of microbial cells disintegration may contribute to the deeper hydrolysis of peptidoglycans, that will increase the yield of target immunotropic muropeptides, reduce the stages number and duration of the process.

The purpose of the work is definition of the rational conditions of enzymatic hydrolysis of the peptidoglycan of *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* biomass cell walls with the participation of papain and lysozyme and, accordingly, the obtaining of the low molecular weight peptides as promising components of immunological functional food ingredients and dietary supplements.

Research objectives:

– to study the patterns of biomass autolysis as the primary stage of peptidoglycans degradation ;

– to determine the rational modes of enzymatic hydrolysis of biomass autolysate with the participation of lysozyme, papain and their combinations, which ensure the maximum accumulation of low molecular weight degradation products of peptidoglycans.

Research materials and methods

Research materials: biomass (BM) of *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* with concentration of $4.8 \cdot 109$ CFU/cm^3 (Ariadna, Odessa); papain with proteolytic activity of 10 U/mg (Swanson Health Products, USA); chicken egg lysozyme, activity> 23,000 Shugar units/mg (Sigma, USA).

Obtaining the degradation products of peptidoglycans Lactobacillus delbrueckii subsp. Bulgaricus B-3964. For pre-disintegration of bacterial cells, their autolysis was performed in the temperature range of 50–70°C, starting from the end of the logarithmic growth phase (8 hours of cultivation) until 48 hours from the beginning of cultivation. The separation of disintegrated cells from the culture fluid was carried out by centrifugation for 15 min at 8000 min^{-1}. The precipitate of the cells was washed with distilled water, resuspended and sent for enzymatic hydrolysis. The enzymatic degradation of BM peptidoglycans was performed by lysozyme and papain separately and in combination. The constant parameters of the hydrolysis were pH=5.5, temperature 37°C. The concentration of lysozyme was varied in the range of 5–10 mg/cm^3, papain in the range of 5–

20 mg/cm^3 and the duration of the process was 0.5–24 h.

In Fig. 1 a, b, c the parametric diagrams are shown which clearly demonstrate the variants of experimental researches. In the first variant (Fig. 1a) to determine the rational parameters of the peptidoglycans destruction in order to obtain low molecular weight peptides, it is necessary to establish the influence of the concentration of lysozyme (C_L, mg/cm^3), the duration of the enzymatic degradation process (τ, h) on the output parameter – the concentration of the low molecular weight peptides in the hydrolysate (C_{LMWP}, mg/cm^3). In the second variant of studies (Fig. 1b), for this purpose it is necessary to establish the effect of papain concentration (C_P, mg/cm^3) and the duration of the hydrolysis process (τ, h) on the concentration of low molecular weight peptides (C_{LMWP}, mg/cm^3). In the third variant (Fig. 1c), to determine the rational parameters of enzymatic hydrolysis, we investigated the effect of papain (C_P, mg/cm^3), lysozyme concentration (C_L, mg/cm^3) and the duration of the process (τ, h) on the concentration of low molecular weight peptides (C_{LMWP}, mg/cm^3).

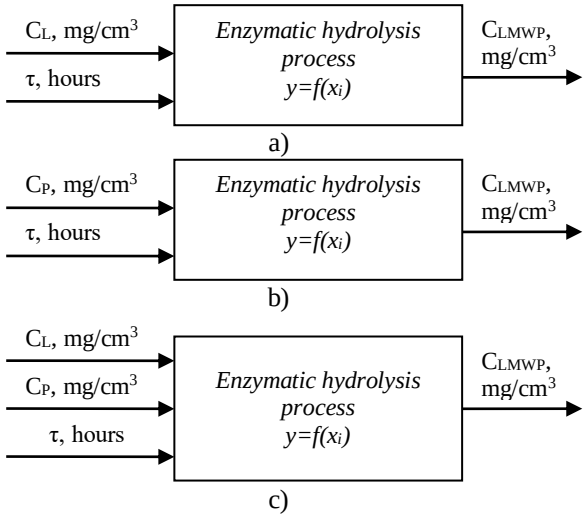


Fig. 1. Parametric schemes of the enzymatic hydrolysis process

The enzymatic hydrolysis was stopped by emergency heating to a temperature of 100°C, the mixture was cooled, the liquid phase was separated from the solid by centrifugation for 10 min at 8000 min^{-1}. In the liquid phase, the content of free amino acids was controlled by formal titration [22]. The content of low molecular weight peptides (LMWP) was determined by the Benedict method [22] after the deposition of high molecular weight proteins with 10% trichloroacetic acid, the content of muropeptides was determined after purification of the hydrolyzate on an ion exchange column with cation exchanger and the subsequent determination of the carbohydrate component in the composition of muropeptides by the Anthrone method [23-24].

In order to reduce the number of experiments and to obtain reliable data on the regularities of the process of enzymatic hydrolysis of BM, the methods of mathematical planning of multifactorial experiments were used. The homogeneity of the results of the experiments was evaluated by Kochren's criterion. To eliminate the effects of systematic errors caused by external conditions and to reduce random errors, experiments were randomized [25,26].

Results of the research and their discussion

Cell walls of microorganisms, especially gram-positive ones, to which lactobacilli belong, have high mechanical strength, which causes a considerable obstacle for their disintegration. The strength of bacterial cells is due to the presence of the structural biopolymer of peptidoglycan in the cell walls. Since it is known that when culturing biomass, cells produce bacterial lysines capable of breaking down specific bonds between peptidoglycan links, the primary destruction of biomass cells was perfomed by autolysis. The course of biomass autolytic changes was studied depending on the duration and temperature of the autolysis process by detecting free amino acids in the culture fluid (Fig. 2).

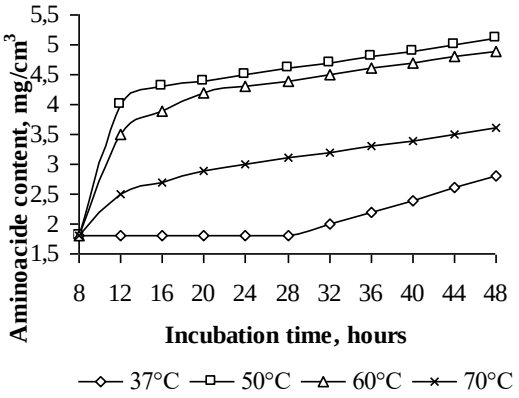


Fig. 2. Dynamics of amino acid accumulation during biomass autolysis

The effect of the degrading effect of autolysis on biomass has been studied in the temperature range of 50–70°C, since it is known that the optimal temperature of the autolysins action is within these limits [20,21]. Incubation according to these regimes was performed after 8th hours from the start of biomass cultivation, which corresponded to the completion of the logarithmic growth phase and, accordingly, the maximum accumulation of bacterial cells [21]. The experiment at 37°C was a control. The most significant autolytic changes in biomass occur at the 12th h of incubation from the beginning of cultivation at 50°C, as evidenced by the maximum accumulation of amino acids in the culture medium (4.2 mg/cm^3), compared with samples that were maintained during the same time under other

temperature modes. Upon further incubation of biomass at 50°C, the concentration of amino acids in the autolysate increases slightly, after 24 h of the process the content of amino acids was 4.5%, after 48 h – 5.1%. The same tendency is observed for other variants of experiments.

The rapid increase in amino acid content in the autolysate at the 12th hour of exposure, apparently due to the fact that at the end of the logarithmic growth phase, bacterial cells are most labile to the action of degrading factors, including autolysins [21]. In the control sample there is a gradual increase in the content of amino acids in the culture medium after 28 hours of cultivation. This course of the process at 37°C is explained by the fact that until the 28th hour of cultivation, the stationary phase of bacterial growth continues [21], which is characterized by a constant value of the content of colony forming units. This practically does not change the composition of the cultural environment. After 28 hours of cultivation, there is a stage of cell death of the biomass, which may be accompanied by an increase in amino acid content. The results of the patterns of *Lactobacillus delbrueckii subsp. Bulgaricus B-3964* biomass autolysis study as the primary stage of peptidoglycans degradation, allow choose the parameters under which the most significant autolytic changes occur, as evidenced by the maximum accumulation of amino acids in the culture medium. Such parameters are incubation of biomass after 8 hours of cultivation at 50°C for 4 hours.

The process of autolysis cannot provide the formation of target low molecular weight peptides, so it is considered only as a factor in the primary destruction of bacteria. For deeper hydrolysis of the peptidoglycans of the biomass autolysate, various variations of enzymatic treatment involving lysozyme, papain and a combination thereof were used. To determine the rational conditions of enzymatic hydrolysis, mathematical modeling of the processes was performed.

It is known that the kinetics of biochemical processes, in particular enzymatic hydrolysis, are described by complex kinetic equations. However, to determine the rational (optimal) conditions for hydrolysis, there is no need to draw up a mathematical description of the entire course of the process of accumulation of the target substance (in our case, low molecular weight peptides). To do this, a mathematical description of only the near-optimal domain will suffice, which will greatly simplify the compilation of both the mathematical model and the solution of the problem of finding extrema (determination of rational conditions). Therefore, in the first stage, a series of search experiments were conducted to study the kinetics of enzymatic processing of BM, which is necessary to determine the rational limits of change of the above factors that affect the process of enzymatic hydrolysis – C_L, C_P та τ.

In Fig. 3. shows the kinetic curves of the process of hydrolysis of BM carried out under different conditions (values of concentrations of C_L and C_P). The graphs show that the process proceeds at different speeds when periods of intense accumulation of LMWPs (increasing concentrations of LMWPs) alternate with less intense periods. It is also seen that the fastest growth of C_{LMWP} lies within the duration of enzymatic hydrolysis of 5–18 h, and the highest values of C_{LMWP} are found on kinetic curves with certain compositions of enzymes (ratios of concentrations of lysozyme C_L and C_P papain). Based on the studies, it was found that further experiments should be carried out in this range of changes in the concentration of enzymes – lysozyme C_L = 5–10 mg/cm^3 and papain C_P = 10–20 mg/cm^3.

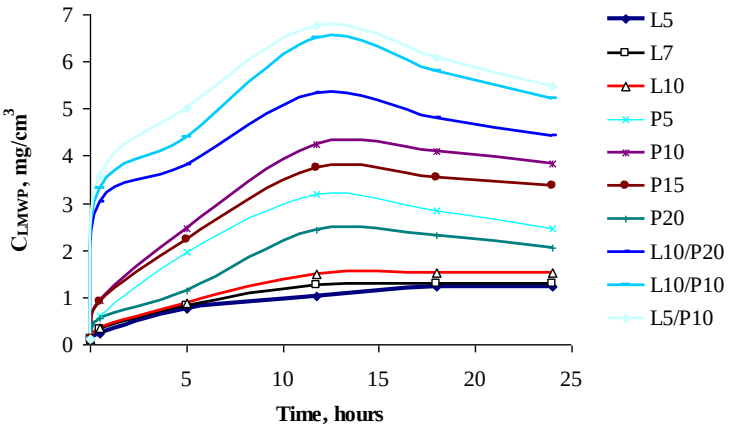


Fig. 3. The kinetics of LMWPs accumulation depending on the type of the BM enzymatic processing and on the duration of the process
(L5, L7, L10 – BM treatment with the lysozyme concentration of 5, 7 and 10 mg/cm^3;
P5, P10, P15, P20 – BM treatment with the papain concentration of 5, 10, 15 and 20 mg/cm^3;
L/P – BM treatment with the enzymes composition lysozyme-papain of appropriate concentration)

The second stage of the research, which was carried out according to the parametric scheme shown in Fig. 1a, was designed to determine the rational conditions for the enzymolysis of BM peptidoglycans

by lysozyme in order to obtain the maximum number of LMWPs. Considering the nonlinearity of the characteristics of the process, the experiments were carried out according to the plan of the second order (the Box type B_2 plan) with an additional point in the center of experiments [25]. The matrix contains nine experiments, the conditions of which are given in Table 1, where the average (in two parallel values) the results of experiments ($C_{LMWP\ av.}$), the calculated values ($C_{LMWP\ cal.}$) by regression equation and the relative errors (δ) between them are given too.

Table 1 – The matrix of the experiment plan and the results of the LMWPs accumulation in the hydrolysate at the using lysozyme

Experiment number	Conditions of the experiments		Results of the experiments		
	C_L, mg/cm^3	τ, hours	$C_{LMWP\ av.}$, mg/cm^3	$C_{LMWP\ cal.}$, mg/cm^3	δ, %
1	5	5	0.75	0.73	3.23
2	10	5	0.88	0.94	7.05
3	5	18	1.23	1.26	2.63
4	10	18	1.51	1.48	2.07
5	5	11.5	1.11	1.14	2.38
6	10	11.5	1.40	1.35	3.38
7	7.5	5	0.83	0.83	0.47
8	7.5	18	1.36	1.37	0.77
9	7.5	11.5	1.26	1.24	1.23

Method of least squares and sequential regression analysis were implemented in the PLAN program [25], than the regression equation in natural variableswe has been obtained, which by Fisher's criterion (F_{cal}=1.66 < F_{cr}=5.14) adequately describes the dependence of C_{LMWP} (mg/cm^3) from the factors C_L and τ.

$$C_{LMWP} = 0{,}04325C_L + 0{,}1187\tau - 0{,}003368\tau^2,$$
$$s=0{,}052.$$

The analysis of the obtained results shows a satisfactory convergence of calculated and experimental data. The relative errors in the experiments are in the range of 0.47–7.05%, the mean square deviation s = 0.04 mg/cm^3, which is quite satisfactory for microbiological and biochemical processes.

It is also seen that the concentration of LMWPs has a direct proportional dependence on C_L and an inverse quadratic dependence on the duration of the enzymatic hydrolysis process τ. The "minus" sign in the quadratic term of the equation indicates that there is a certain value of τ that gives the maximum accumulation of C_{LMWP}.

Using the obtained equation and the PLAN program, two-factor dichotomy method was used to determine the rational calculated values of the factors C_L and τ (within the studied limits of their change), which provide the maximum concentration of low molecular weight peptides C_{LMWP}: C_L=10 mg/cm^3, τ =17,63 hours, C_{LMWP} =1,48 mg/cm^3.

These calculated hydrolysis conditions are close to those of Experiment 4, in which the experimental concentration value was 1.51 mg/cm^3, which is close to the calculated one. More clearly the nature of the influence of the factors C_L and τ on the accumulation process of low molecular weight in the hydrolysate can be seen from the graphical interpretation of the regression equation, which is shown in Fig. 4.

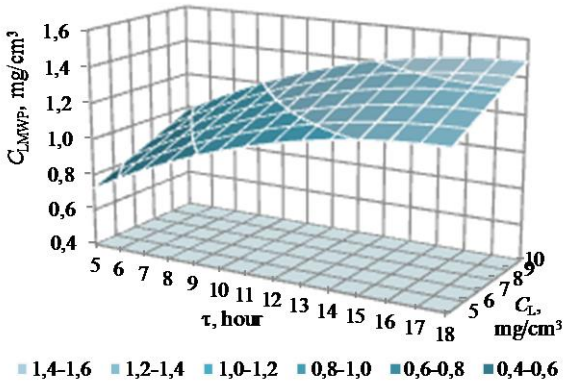


Fig. 4. The response surface of C_{LMWP} depending on factors C_L and τ

According to the parametric scheme on Fig. 1b, the second series of researches has been realized by the second order Box plan of the B_2 type with the central point. The conditions of the experiments, the values of the factors C_P and τ, the results of enzymatic hydrolysis with papain are given in table 2.

The regression equation in natural variableswe has been obtained after the experiments, which by Fisher's criterion (F_{cal} = 1,42 <F_{cr} = 6,94) adequately describes the dependence of C_{LMWP} (mg/cm^3) on the factors C_p and τ:

$$C_{LMWP} = -2.081 + 0.4450C_P + 0.5337\tau - 0.01967C_P^2 -$$
$$-0.01945\tau^2, \quad s=0.14.$$

Table 2 – The matrix of the experiment plan and the results of the LMWPs accumulation in the hydrolysate at the using papain

Experiment numbe	Conditions of the experiments		Results of the experiments		
	C_P, mg/cm^3	τ, hours	$C_{LMWP\ av.}$, mg/cm^3	$C_{LMWP\ cal.}$, mg/cm^3	δ, %
1	5	5	1.95	1.83	5.93
2	20	5	1.14	1.13	0.49
3	5	18	2.84	2.96	4.15
4	20	18	2.31	2.26	2.26
5	5	11.5	3.22	3.22	0.07
6	20	11.5	2.46	2.51	2.35
7	15	5	2.23	2.35	5.43
8	15	18	3.54	3.47	1.85
9	15	11.5	3.79	3.73	1.47

Results of processing of the experimental data show satisfactory convergence of calculated and experimental data. The relative errors in the experiments are in the range of 0.07–5.93%, the standard deviation "s" is 0.12 mg/cm^3, which is quite satisfactory.

From the obtained equation it is seen that the concentration of C_{LMWP} has an inverse parabolic dependence on the concentration of papain C_p and the duration of the hydrolysis process τ. The sign "minus" of the quadratic terms of the equation indicates the existence of the maximum accumulation of C_{LMWP} under certain conditions of enzymatic hydrolysis. Based on the obtained equation, the rational values of the factors C_p and τ were determined, which provide the highest concentration of low molecular weight peptides C_{LMWP}:

$$C_P = 11.31 \text{ mg/cm}^3,\ \tau = 13.72 \text{ hours},$$
$$C_{LMWP} = 4.10 \text{ mg/cm}^3.$$

As can be seen, the use of papain instead of lysozyme allows to increase the concentration of low molecular weight peptides C_{LMWP} in 2.8 times under certain rational conditions. Peculiarities of the C_P and τ influence factors on the process of low molecular weight peptides accumulation C_{LMWP} are shown in Fig. 5, which was built on the basis of the above equation.

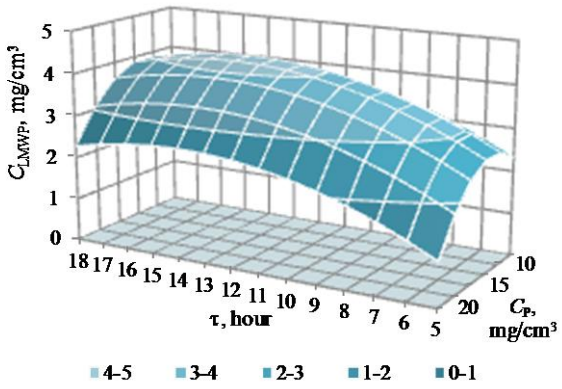


Fig. 5. The response surface of C_{LMWP} depending on factors C_p and τ

Finally, the regularities of enzymatic hydrolysis of peptidoglycans by the enzymes combination lysozyme: papain were studied. The experiments were performed according to the parametric scheme in Fig. 1c, by the second order Box plan of the B_3 type containing 15 experiments. The levels of factors and conditions of experiments and their results are given in table 3.

After processing the results of the experiments using the PLAN program, the regression equation in natural variables was obtained. Statistical evaluation of this equation by Fisher's criterion (F_{cal}= 1,13 < F_{cr} = 19,37) showed its adequacy, which allows to use it to describe the dependence of the concentration of C_{LMWP} (mg/cm^3) on the factors C_L, C_p and τ, as well as to justify rational conditions for enzymatic hydrolysis. The resulting equation is as follows:

$$C_{LMWP} = 2{,}637 - 0{,}8075C_L + 0{,}3975C_P + 0{,}6818\tau + 0{,}05191C_L^2 - 0{,}01702C_P^2 - 0{,}02581\tau^2,\ \ s=0{,}11.$$

It can be seen from table. 3, the relative errors in the experiments are in the range of 0.08–4.16%, the standard deviation is 0.11 mg/cm^3, which shows a satisfactory convergence of the calculated and experimental data and is quite acceptable.

Analysis of the regression equation shows a nonlinear (parabolic) dependence of the concentration of low molecular weight peptides C_{LMWP} in the hydrolysate on the concentration of lysozyme C_L, papain C_P and the duration τ of enzymatic hydrolysis of BM peptidoglycans.

The sign "minus" for the quadratic terms C_P^2 and τ^2 indicates the existence of extreme concentrations (maxima) of SNMP for certain values of these factors. The coefficient at C_L^2 has a "plus" sign, which indicates the possibility of increasing the concentration of LMWPs C_{LMWP} both with increasing and decreasing the concentration of lysozyme C_L at a certain value that coincides with the axis of the parabola.

Based on the obtained equation, calculations were performed according to the PLAN program, which realizes the search for function extremes by the method of multidimensional dichotomy, and the conditions of maximum accumulation (concentration) of low molecular weight peptides C_{LMWP} were determined.

Rational conditions for enzymatic hydrolysis of BM peptidoglycans, ie the values of factors C_L, C_P and τ, as

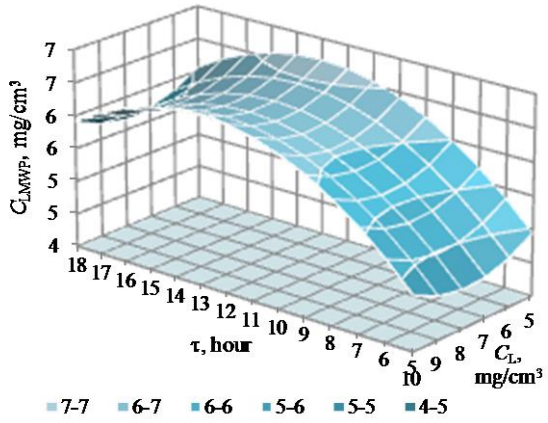
well as the maximum calculated value of C_{LMWP}, which can be achieved within certain limits of research, are as follows:

C_L = 5,00 mg/cm^3, C_P = 11,67 mg/cm^3,
τ = 13,21 hour, C_{LMWP} = 6,72 mg/cm^3.

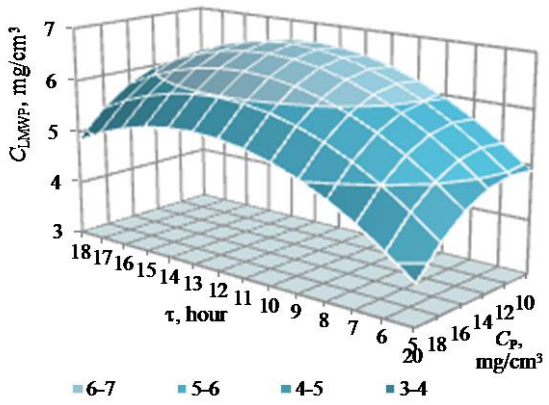
Visually, the nature of the pairwise effect of factors C_L, C_P and τ on the process of accumulation of low molecular weight peptides C_{LMWP} in the hydrolysate is shown in Fig. 6 in the form of response surfaces. The values of the third factors were taken at the extreme (rational) levels shown in the figures.

Table 3 – The matrix of the experiment plan and the results of the LMWPs accumulation in the hydrolysate at the using enzyme composition lysozyme : papain

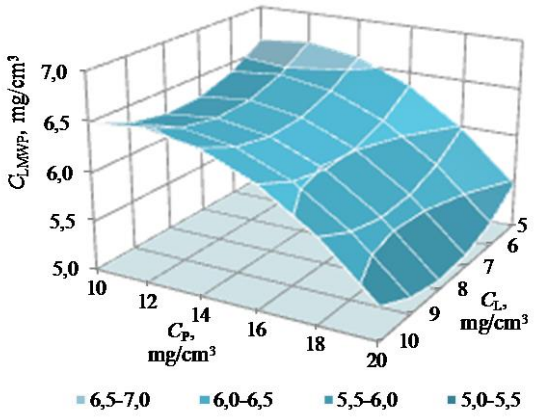
Experiment numbe	Conditions of the experiments			Results of the experiments		
	C_L, mg/cm^3	C_P, mg/cm^3	τ, hours	$C_{LMWP\ av.}$, mg/cm^3	$C_{LMWP\ cal.}$, mg/cm^3	δ, %
1	5	10	5	5.00	4.93	1.32
2	10	10	5	4.68	4.79	2.35
3	5	20	5	3.65	3.80	4.16
4	10	20	5	3.80	3.66	3.74
5	5	10	18	6.21	6.08	2.10
6	10	10	18	5.88	5.94	0.95
7	5	20	18	4.94	4.95	0.16
8	10	20	18	4.80	4.80	0.08
9	5	15	11.5	6.42	6.46	0.57
10	10	15	11.5	6.34	6.31	0.43
11	7.5	10	11.5	6.17	6.20	0.50
12	7.5	20	11.5	5.09	5.07	0.41
13	7.5	15	5	4.45	4.40	1.19
14	7.5	15	18	5.48	5.54	1.15
15	7.5	15	11.5	6.08	6.06	0.32



a) C_P = 5,00 mg/cm^3



b) C_L = 11,67 mg/cm^3



c) τ = 11,67 hour

Fig. 6. The response surfaces of C_{LMWP} depending on factors C_L, C_p and τ

Experimental verification of the calculated rational conditions of the enzymatic hydrolysis process confirmed the possibility of obtaining the maximum accumulation (concentration) of low molecular weight peptides C_{LMWP}. Under certain process conditions, the average accumulation of LMWPS in the hydrolyzate was 6.58 mg/cm^3. Using the previously developed method [23], the content of target muropeptides in the LMWPs was determined, which was 4.35 mg/cm^3, which significantly exceeds the existing studies [13,14] and proves the effectiveness of the developed method of peptidoglycan destruction.

Thus, the use of the composition of lysozyme and papain will allow for the ratio of their concentrations as 1 : 2.33 and duration of fermentolysis 13 h 13 min to obtain the highest concentration of SNMP, and therefore the highest yield of low molecular weight peptides in the process of destruction of peptidoglycans BM.

When comparing the variations of the enzymatic treatment, the use of the hydrolase composition for peptidoglycan degradation is 40% more effective than papain treatment and 80% more effective than lysozyme treatment, as evidenced by the concentration of LMWPs in the reaction media. This difference can be explained by the fact that for deep hydrolysis of peptidoglycan, a prerequisite is the use of two types of hydrolases, namely, muramidase (lysozyme), which hydrolyzes β 1→4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine, (papain), which catalyzes the rupture of peptide bonds of peptides that connect lactic acid residues of formic acid in parallel chains consisting of alternating blocks of N-acetylmuramic acid and N-acetylglucosamine [17].

The use of papain as a protease determines the possibility of simultaneous treatment of the autolysate with the composition lysozyme-papain, because the pH optimum of these hydrolases is in one range [18]. Due to this, there is a reduction in the duration of the process of LMWPs obtaining and increase its efficiency, because in experiments with sequential treatment by enzymes, the content of LMWPs in the reaction medium is 10–15% less than during simultaneous processing (Patent of Ukraine UA 138938 The method of obtaining a glycopeptide product from the cell walls of probiotic bacteria, 2019). That may indicate a synergistic effect of these enzymes in the presence of each other.

According to the calculated rational parameters of fermentolysis with the combination of enzymes (C_L = 5.00 mg/cm^3, C_P = 11.67 mg/cm^3, τ = 13.21 h), an experiment was performed in which biomass autolysis was not used as the primary stage of destruction. As a result, the content of NMP in the reaction medium was 4.83 mg/cm^3, which is 27% less than in the experiment involving autolysis. Thus, the autolysis stage is necessary in obtaining low molecular weight fragments of peptidoglycans of probiotic cultures, as it significantly increases the depth of further enzymatic hydrolysis.

Conclusion

The regularities of biomass autolysis as the primary stage of peptidoglycan destruction were studied and it was determined that the most significant autolytic changes occur during biomass incubation after 8 hours of cultivation at 50°C for 4 hours, as evidenced by a sharp increase in amino acid content in culture medium (4,2 mg/cm^3).

The range of concentrations of lysozyme C_L = 5–10 mg/cm^3 and papain C_P = 10–20 mg/cm^3, which provide maximum accumulation of low molecular weight peptidoglycan degradation products, was determined.

Rational regimes of enzymatic hydrolysis of biomass autolysate with the participation of lysozyme, papain and their combination were determined by the method of mathematical modeling of multifactor experiments. These regimes provide maximum accumulation of low molecular weight peptidoglycan degradation products.

The least squares method and sequential regression analysis implemented in the PLAN program yielded regression equations in natural variables, which, according to Fisher's criterion, adequately describe the dependence of the output factor on the input ones. Rational parameters of enzymatic hydrolysis with lysozyme (C_L = 10 mg/cm^3, τ = 17.63 h, C_{LMWP} = 1.48 mg/cm^3), with papain (C_P = 11.31 mg/cm^3, τ = 13.72 h, C_{LMWP} = 4.10 mg/cm^3) and with a combination of enzymes (C_L = 5.00 mg/cm^3, C_P = 11.67 mg/cm^3, τ = 13.21 h, C_{LMWP} = 6.72 mg/cm^3) were determined.

References:

1. Franca TGD et al. Impact of malnutrition on immunity and infection. J Venom Anim Toxins incl Trop Dis. 2009;15(3):375. https://doi.org/10.1590/S1678-91992009000300003
2. Simon AK, Hollander GA, McMichael A. 2015 Evolution of the immune system in humans from infancy to old age. Proc. R. Soc. B 282: 20143085. http://dx.doi.org/10.1098/rspb.2014.3085
3. Petter Brodin and Mark M. Davis Human immune system variation Nat Rev Immunol. 2017 January;17(1):21-29. doi:10.1038/nri.2016.125.
4. Biggest Threats and Data. URL https://www.cdc.gov/drugresistance/biggest-threats. html
5. Vavricka SR et al. hPepTl transports muramyl dipeptide, activating NF -kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. Gastroenterology. 2004;127:1401-1409. https://doi.org/10.1053/j.gastro.2004.07.024
6. Traub S. MDP and other muropeptides – direct and synergistic effects on the immune system. J. Endotoxin Res. 2006;12(2):69-85. https://doi.org/10.1179/096805106X89044
7. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. Nat. Immunol. 2010;11:373-384. https://doi.org/10.1038/ni.1863

8. Matsui K, Ikeda R. Peptidoglycan in combination with muramyldipeptide synergistically induces an interleukin-10-dependent T helper 2-dominant immune response. Microbiol Immunol. 2014;58:260-265. https://doi.org/10.1111/1348-0421.12139
9. Matsumoto S et al. A component of polysaccharide peptidoglycan complex on *Lactobacillus* induced an improvement of murine model of inflammatory bowel disease and colitis-associated cancer. Immunology. 2009;128:170-80. https://doi.org/10.1111/j.1365-2567.2008.02942.x
10. Kuhner D, Stahl M, Demircioglu DD, Bertsche U. From cells to muropeptide structures in 24 h: Peptidoglycan mapping by UPLC-MS. Sci. Rep. 2014;4:74-94. https://doi.org/10.1038/srep07494
11. Irazoki O, Hernandez SB, Cava F. Peptidoglycan Muropeptides: Release, Perception, and Functions as Signaling Molecules. Front. Microbiol. 2019;10:500. http://dx.doi.org/10.3389/fmicb.2019.00500
12. Livinskaya EP, Kovalenko NK, Garmasheva IL. Dezintegraciya laktobacill i enterokokkov dlya polucheniya fragmentov kletochnyh stenok.Mikrobiologichnij zhurnal. 2011; 73(3): 26-32.
13. Gavrilin MV, Senchukova GV, Senchenko SP. Vybor optimalnyh uslovij polucheniya gidrolizatov molochnokislyh bakterij termokislotnym sposobom. Him.-farm. zhurn. 2007;41(2):54-56.
14. Senchenko SP et al. Izuchenie sostava preparata, poluchennogo na osnove gidrolizata molochnokislyh bakterij. Him.-farmac. zhurn. 2005;39(3):51-53
15. Erickson KL, Hubbard NE. Probiotic immunomodulation in health and disease. J Nutri. 2000;130:403-409. https://doi.org/10.1093/jn/130.2.403S
16. Glushanova NA. Biologicheskie svojstva laktobacill. Byulleten sibirskoj mediciny. 2003;4:50-58
17. Chapot-Chartier M-P, Kulakauskas S. Cell wall structure and function in lactic acid bacteria. Microbial Cell Factories. 2014;13(Suppl 1):S9:1-23. https://doi.org/10.1186/1475-2859-13-S1-S9.
18. Ovsyannikova LV, Komarova EL. Sravnitelnaya harakteristika proteoliticheskih fermentov rastitelnogo proishozhdeniya – papaina i bromelajna. BAD. 2012;7(74):26-32. https://doi.org/10.7748/ns2012.01.26.19.32.p7281
19. Regulski K. Analysis of the Peptidoglycan Hydrolase Complement of Lactobacillus casei and Characterization of the Major c-D-Glutamyl-L-Lysyl-Endopeptidase. PLoS ONE. 2012;7(2):e32301. https://doi.org/10.1371/journal.pone.0032301
20. Martinez-Cuesta M-C et al. Requirement of Autolytic Activity for Bacteriocin-Induced Lysis. Applied and environmental microbiology. 2000;Aug.:3174-3179. https://doi.org/10.1128/AEM.66.8.3174-3179.2000
21. Simova E, Beshkova D. Effect of growth phase and growth medium on peptidase activities of starter lactic acid bacteria. Le Lait, INRA Editions. 2007;87(6):555-573. https://doi.org/10.1051/lait:2007036
22. Semak IV, Zyryanova TN, Gubich OI..Biohimiya belkov: praktikum dlya studentov biol. Fak. spec. 1-31 01 01 «Biologiya». Minsk: BGU; 2007.
23. Kapustian AI, Cherno NK, Pukas AS. Rozroblennia metodu vyznachennia funktsionalnykh kharchovykh inhrediientiv muropeptydnoho pokhodzhennia u bakterialnykh dezintehratakh. Naukovi pratsi NUKhT. 2019; 25(6):157-164. https://doi.org/10.24263/2225-2924-2019-25-6-21
24. Morris DL. Quantitative Determination of Carbohydrates With Dreywood's Anthrone Reagent. Science. 1948;107(2775):254-5. https://doi.org/10.1126/science.107.2775.254
25. Ostapchuk NV, Kaminskij VD, Stankevich GN, Chuchuj VP. Matematicheskoe modelirovanie processov pishevyh proizvodstv: Sb. zadach: Uch. Posobie. K.: Visha shkola; 1992.
26. Voznesenskij VA. Statisticheskie metody planirovaniya eksperimenta v tehniko-ekonomicheskih issledovaniyah. M.: Finansy i statistika; 1981.

ВИЗНАЧЕННЯ РАЦІОНАЛЬНИХ РЕЖИМІВ ОТРИМАННЯ НИЗЬКОМОЛЕКУЛЯРНИХ ПЕПТИДІВ ІЗ ПЕПТИДОГЛІКАНІВ *Lactobacillus delbrueckii subsp. Bulgaricus*

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Анотація. Проблему зниження імунного статусу населення доцільно коригувати за допомогою нутрітивної підтримки. Складові пептидогліканів клітинних стінок пробіотичних бактерій (низькомолекулярні муропептиди) – перспективні компоненти функціональних харчових інгредієнтів та дієтичних добавок, що володіють потужною імунотропною активністю. У роботі запропоновано отримання цільових пептидів за допомогою комбінування автолізу та ферментативного гідролізу пептидогліканів біомаси *Lactobacillus delbrueckii subsp. Bulgaricus B-3964.* Вплив деградуючої дії автолізу на біомасу вивчали в інтервалі температур 50–70°С, інкубування за даних режимів проводили після 8-ми годин від початку культивування. Встановлено, що найбільш значні автолітичні зміни відбуваються при інкубуванні біомаси після 8-ї години культивування при 50°С протягом 4-х год, про що свідчить різке збільшення вмісту амінокислот у культуральному середовищі (4,2 мг/см^3). Для більш глибокого гідролізу пептидогліканів автолізату біомаси використовували різні варіації ферментативної обробки за участю лізоциму, папаїну та їхньої комбінації. Для визначення раціональних умов ферментолізу застосовували методи математичного планування багатофакторних експериментів, в якому вхідними факторами були: C_L – концентрація лізоциму, мг/см^3; C_P, – концентрація папаїну, мг/см^3; τ – тривалість процесу, год. Вихідний параметр математичного моделювання – концентрація низькомолекулярних пептидів у ферментолізаті (C_{LMWP}, мг/см^3). Проведено серію експериментів з дослідження особливостей ферментативного гідролізу біомаси з метою визначення раціональних меж вхідних факторів. З'ясовано, що максимальна концентрація низькомолекулярних пептидів $C_{НМП}$ у ферментолізаті має місце у певних діапазонах концентрацій ферментів: лізоциму C_L = 5–10 мг/см^3, папаїну C_P = 10–20 мг/см^3. Методом найменших квадратів та послідовного регресійного аналізу, реалізованих у програмі PLAN, отримали рівняння

регресії у натуральних змінних, які за критерієм Фішера адекватно описують залежність вихідного фактору від вхідних. Визначено раціональні параметри ферментолізу лізоцимом (C_L=10 мг/см^3, τ =17,63 год, $C_{НМП}$ =1,48 мг/см^3), папаїном (C_P = 11,31 мг/см^3, τ = 13,72 год, $C_{НМП}$ = 4,10 мг/см^3) та комбінацією ферментів (C_L = 5,00 мг/см^3, C_P = 11,67 мг/см^3, τ = 13,21 год, $C_{НМП}$ = 6,72 мг/см^3). Вміст цільових муропептидів при цьому складає 4,35 мг/см^3

Ключові слова: низькомолекулярні пептиди, пептидоглікан, пробіотичні бактерії, автоліз, ферментативний гідроліз, лізоцим, папаїн, математичне моделювання.

Список літератури:

1. Impact of malnutrition on immunity and infection / Franca T.G.D et al. // J Venom Anim Toxins incl Trop Dis. 2009. Vol. 15(3). P. 374-390. https://doi.org/10.1590/S1678-91992009000300003
2. Simon A.K., Hollander G.A., McMichael A. Evolution of the immune system in humans from infancy to old age // Proc. R. Soc. 2015. B 282. 20143085. http://dx.doi.org/10.1098/rspb.2014.3085
3. Brodin P., Davis Mark M. Human immune system variation // Nat Rev Immunol. 2017 January. Vol. 17(1). P. 21-29. https://doi.org/10.1038/nri.2016.125.
4. Biggest Threats and Data. Веб-сайт. URL https://www.cdc.gov/drugresistance/biggest-threats. html: (дата звернення 10.09.2019)
5. Vavricka S.R. et al. hPepTl transports muramyl dipeptide, activating NF -kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells // Gastroenterology. 2004. V. 127. P. 1401-1409. https://doi.org/10.1053/j.gastro.2004.07.024
6. Traub S. MDP and other muropeptides – direct and synergistic effects on the immune system // J. Endotoxin Res. 2006. V. 12(2). P. 69-85. https://doi.org/10.1179/096805106X89044
7. Kawai T., Akira S. The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors // Nat. Immunol. 2010. V. 11. P. 373-384. https://doi.org/10.1038/ni.1863
8. Matsui K., Ikeda R. Peptidoglycan in combination with muramyldipeptide synergistically induces an interleukin-10-dependent T helper 2-dominant immune response // Microbiol Immunol. 2014. V. 58, P. 260–265. https://doi.org/10.1111/1348-0421.12139
9. Matsumoto S. et al. A component of polysaccharide peptidoglycan complex on *Lactobacillus* induced an improvement of murine model of inflammatory bowel disease and colitis-associated cancer // Immunology. 2009. V. 128. P. 170-80. https://doi.org/10.1111/j.1365-2567.2008.02942.x
10. Kuhner D., Stahl M., Demircioglu D.D., Bertsche U. From cells to muropeptide structures in 24 h: Peptidoglycan mapping by UPLC-MS // Sci. Rep. 2014. V. 4. P. 74-94. https://doi.org/10.1038/srep07494
11. Irazoki O., Hernandez SB, Cava F. Peptidoglycan Muropeptides: Release, Perception, and Functions as Signaling Molecules // Front. Microbiol. 2019;10:500. http://dx.doi.org/10.3389/fmicb.2019.00500
12. Ливинская Е.П., Коваленко Н.К., Гармашева И.Л. Дезинтеграция лактобацилл и энтерококков для получения фрагментов клеточных стенок // Мікробіологічний журнал. 2011. Том 73. №3. С. 26-32.
13. Гаврилин М.В., Сеньчукова Г.В., Сенченко С.П. Выбор оптимальных условий получения гидролизатов молочнокислых бактерий термокислотным способом // Хим.-фарм. журн. 2007. Т. 41, № 2. С. 54–56.
14. Сенченко С.П., Самойлов В.А., Гостищева Н.М., Сеньчукова Г.В., Гаврилин М.В. Изучение состава препарата, полученного на основе гидролизата молочнокислых бактерий // Хим.-фармац. журн. Т.39, №3. 2005. С. 51–53
15. Erickson K.L., Hubbard N.E. Probiotic immunomodulation in health and disease // J Nutri. 2000. V. 130. P. 403-409. https://doi.org/10.1093/jn/130.2.403S
16. Глушанова Н.А. Биологические свойства лактобацилл // Бюллетень сибирской медицины. 2003. №4. С. 50-58
17. Chapot-Chartier M.-P., Kulakauskas S. Cell wall structure and function in lactic acid bacteria // Microbial Cell Factories. 2014. 13(Suppl 1):S9. P.1-23. https://doi.org/10.1186/1475-2859-13-S1-S9.
18. Овсянникова Л.В., Комарова Е.Л. Сравнительная характеристика протеолитических ферментов растительного происхождения – папаина и бромелайна // БАД. 2012. №7 (74). С. 26-32. https://doi.org/10.7748/ns2012.01.26.19.32.p7281
19. Analysis of the Peptidoglycan Hydrolase Complement of Lactobacillus casei and Characterization of the Major c-D-Glutamyl-L-Lysyl-Endopeptidase / Regulski K. Et al. // PLoS ONE. 2012. V. 7(2). e32301. https://doi.org/10.1371/journal.pone.0032301
20. Requirement of Autolytic Activity for Bacteriocin-Induced Lysis / Martinez-Cuesta M.-C. et al. // Applied and environmental microbiology. 2000. Aug., P. 3174-3179. https://doi.org/10.1128/AEM.66.8.3174-3179.2000
21. Simova E., Beshkova D. Effect of growth phase and growth medium on peptidase activities of starter lactic acid bacteria // Le Lait, INRA Editions. 2007. V. 87 (6). P. 555-573. https://doi.org/10.1051/lait:2007036
22. Биохимия белков: практикум для студентов биол. Фак. спец. 1-31 01 01 «Биология» / И. В. Семак, Т. Н. Зырянова, О. И. Губич. Минск: БГУ, 2007. 49 с.
23. Капустян А. І., Черно Н. К., Пукас А. С. Розроблення методу визначення функціональних харчових інгредієнтів муропептидного походження у бактеріальних дезінтеграtах // Наукові праці НУХТ. 2019. Том 25, № 6. С. 157-164. https://doi.org/10.24263/2225-2924-2019-25-6-21
24. Morris D.L. Quantitative Determination of Carbohydrates With Dreywood's Anthrone Reagent. Science. 1948. Vol. 107(2775). P. 254-5. https://doi.org/10.1126/science.107.2775.254
25. Остапчук Н.В., Каминский В.Д., Станкевич Г.Н., Чучуй В.П. Математическое моделирование процессов пищевых производств: Сб. задач: Уч. Пособие. К.: Вища школа, 1992. 175 с.
26. Вознесенский В.А. Статистические методы планирования эксперимента в технико-экономических исследованиях. М.: Финансы и статистика, 1981 262 с.