UDC 579.852.11.24 https://doi.org/10.15407/biotech11.02.040

MATHEMATICAL MODELLING OF OPTIMIZATION NUTRIENT MEDIUM COMPOSITION FOR ENTOMOPATHOGENIC BACTERIA STRAIN *Bacillus thuringiensis* 87/3 CULTIVATION

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Received 03.01.2018

The aim of the research was to develop an optimal liquid medium composition for bacteria strain *B. thuringiensis* 87/3 which is the most favorable for the identification the potential production of biologically active components. In this work we have used new entomopathogenic bacteria strain *B. thuringiensis var. thuringiensis (Bt H1)* № 87/3 selected *in vitro*, isolated from the larvae of leaf-eating insects natural populations of *Leptinotarsa decemlineata* Say (L_4). Nutrient medium for cultivation of *Bt* 87/3 was optimized on base a full-fledged experiment, according to the Box-Behnken (3^3) plan. Optimumal concentrations were investigated in the medium of carbon source (15 g/l of glucose), nitrogen (10 g/l of corn extract) and phosphorus-containing inorganic salts (1,5 g/l of diammonium phosphate). When this strain grown in periodic conditions at 30 °C, the number of viable cells reached $4{,}4 \cdot 10^9$ cells/ml. Nutrient medium that was optimized can be recommended for cultivation of *Bacillus thuringiensis* 87/3 in production conditions.

Key words: optimization, cultivation, *B. thuringiensis var. thuringiensis*, nutrient medium.

Biological pesticides is one of the most promising alternatives over conventional chemical pesticides, which offers less or no harm to the environments and biota. A large range of microorganisms such as bacteria, viruses, fungi, and protozoans have since been identified as potential candidates for use in biocontrol strategies against insect pests. The dominant position among the complex of entomopathogenic microorganisms known in the world and used in the protection of plants occupy entomopathogens belonging to the species *Bacillus thuringiensis (Bt)*[1,2]. There are more than 80 serovariants of *Bt*, selectively specific to the definite groups of host insects belonging to the order *Lepidoptera*, *Diptera, Coleoptera, Hymenoptera, Orthoptera, Hemiptera, Isoptera, Mallophaga, Thisanoptera, Nematoda, Acari* [3].

Serological *Bt* variants produce different entomotoxins, their synthesis in many respects depends on the conditions of cultivation. Toxigenicity of microorganisms can be changed by biotechnological procedures (changing the conditions of cultivation) and thus affect metabolism in general [4]. The most important stage in the production of bacterial preparations is obtaining the maximum production of delta endotoxin in a minimum time of cultivation with a maximum economic effect.

The aim of the research was to develop an optimal liquid medium composition for bacteria strain *B.thuringiensis 87/3*, which are the most favorable for the identifiying the potential production of biologically active components.

Materials and Methods

Research was conducted on the base of the National University of Life and Environmental Sciences of Ukraine, department of biotechnology and biodiversity; Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine, department of industrial and food biotechnology. The subject of researches

was entomopathogenic bacteria strain *B. thuringiensis var. thuringiensis (Bt H1) №87/3* selected in vitro, isolated from the larvae of leaf-eating insects natural populations of *Leptinotarsa decemlineata* Say (L_4). The qualitative and quantitative composition of medium components was determined on the basis of a priori information analysis.

The task of full-fledged experiment, according to the Box-Behnken (3^3) plan is based on obtaining a mathematical model of the *Bacillus thuringiensis 87/3* development process and its subsequent use in nutrient medium optimizing.

The planning of an experiment allows not only to vary all the operating factors during the process, but also to obtain a quantitive assessment of the main factors and the effects of interaction between them [5]. Optimization is possible using methods of steep climbing, as well as research of the desirability function of the resulting factor. The influence of different medium composition on the growth of microorganisms was investigated during active experiment process. The average level for *Bacillus thuringiensis 87/3* cultivating was corn extract 10 g/l, diammonium phosphate 1.5 g /l, glucose 15 g/l (Table 1).

Cultivation was carried out in Erlenmeyer's flasks on biotechnology shaker with termoplatform (220 rev./min, the temperature 30 degrees °C during 72 hours). Medium, of volume 50 ml, the amount of inoculum — at least 4.0% by volume of the medium (the titer of colony forming units, CFU, 4.2–4.4 billion/ml of the culture liquid, which was determined by inoculation on agar and counting in Goryaev chamber).

For the experiment conduction it was decided to investigate three factors and their three levels. In this case, the number of experiments that must be carried out can be calculated by the formula (1):

$$N_e = (N_\Phi)^p, \qquad (1)$$

where N_e — the number of experiments, p — the number of levels factors.

Since formula (1) shows that 27 experiments are needed to solve optimization problems. The plan for a full-fledged experiment with the specified levels and factors, as well as the function of the experiment response, has been formed. In order to reduce the impact on the results of the response, the experiments were carried out in random sequence. The processing of the experiment results began with regression analysis: the model was built and unknown coefficients were determined:

$$Y = (b_0 + b_1 \cdot X1 + b_2 \cdot (X1)^2 + b_3 \cdot X2 + \\ + b_4 \cdot (X2)^2 + b_5 \cdot X3 + b_6 \cdot (X3)^2) \cdot 10^9 \qquad (2)$$

It was determined that the effects of the interaction factors are practically absent, and therefore they were not included in the general view of the model (2). The determination of unknown constant coefficients was performed using the least squares method. The obtained model coefficients were calculated using the following formulas:

$$b_0 = \sum_{i=1}^{N} Y_i / N; \quad b_j = \sum_{U=1}^{N} Y_i X_{ji} / N;$$

$$b_{j^2} = \sum_{i=1}^{N} Y_i (X_{ji})^2 / N.$$

Description of factors and response using a mathematical model (2) is characterized by a determination coefficient, which must be not less than 0.95, for qualitative description of the research object, this coefficient is calculated by the formula,

$$R^2 = 1 - \frac{\sigma^2_{з.р.}}{\sigma^2_Y} = 1 - \left(\sum_{i=1}^{N} (Y_i - \hat{Y}_i)^2 / \sum_{i=1}^{N} (Y_i - \bar{Y})^2\right), \qquad (3)$$

where $\sigma_{з.р.}{}^2$, $\sigma_Y{}^2$ — dispersion of residues regression, response; Y_i, $\bar{Y}$, $\hat{Y}_i$ — actual, average, estimated value of response.

Table 1. **Formation of factors and their levels for the experiment**

Factors	Levels			
	Lower	Main	Upper	Variation Step
X1 — corn extract, g/l	5	10	15	5
X2 — diammonium phosphate, g/l	0,5	1,5	2,5	1
X3 — glucose, g/l	5	15	25	10

The standard error which characterizing the standard deviation of the studied regression coefficients from the mean value is calculated by the formula:

$$S_{b_{j^r}} = \sqrt{\frac{\sum_{i=1}^{N}(Y_i - \hat{Y}_i)^2}{\sum_{i=1}^{N}(X_j - \bar{X}_j)^2} \cdot \frac{1}{n-2}}, \quad (4)$$

where n — sample size.

The statistical significance of the regression coefficient is estimated according to Student's criterion. In this case, compare the calculated with the table value for a given confidence level significance of 0.05 and freedom degrees calculated:

$$\left|t_{\alpha,f}\right| = \left|\frac{b_{j^r}}{S_{b_{j^r}}}\right| \geq t_{\alpha/2,f.table,} \quad (5)$$

where b_{jr} — estimated regression coefficients, α — confidence probability 0.95, f — freedom degree. With a significant regression coefficient, the student's calculated criterion is over than tabular.

The calculation of the marginal deviation error was established from the following calculations:

$$\Delta_{jr} = t_{\alpha,f.table} \cdot S_{b_{jr}} \quad (6)$$

Determination of the confidence interval for each regression coefficient was conducted according to the inequality:

$$b_{jr} - \Delta_{jr} \leq b_{jr} \leq b_{jr} + \Delta_{jr} \quad (7)$$

The correspondence of the mathematical model to the experimental data was determined by Fisher's criterion (F). In this case, the calculation criterion should be over than the tabular one:

$$F = \frac{\sigma_X^2}{\sigma_Y^2} = \frac{R^2}{1-R^2} \cdot \frac{f_2}{f_1} \geq F_{\alpha,f.table,}$$

where $\sigma_X^2 = \left(\sum_{i=1}^{N}(X_i - \bar{X})^2\right)/f_1$ — variance factor; $f_1 = k_b$ — freedom degree; k_b — the coefficients number of the regression model; $\sigma_Y^2 = \left(\sum_{i=1}^{N}(Y_i - \bar{Y})^2\right)/N - f_1 - 1$ — response variance, N — number of experiments, R^1 — determination factor.

To solve the optimization problem, the method of analyzing the desirability function of Harrington was used [6].

The analysis of this experiment plan was carried out using portable software (Statistica 10.0.1011.0, CD-key 42347678921334567692).

Results and Discussion

Bacillus thuringiensis grows slowly and spores weakly on media with a known composition containing glucose and salts. Growth can be enhanced by the addition of amino acids or casein hydrolyzate, but if not balanced the nitrogen medium content with an appropriate source of carbon and energy, such as glucose, sporulation will remain low.

Optimization of cultivation conditions can be based on a combination of experimental and mathematical modeling with a computational experiment, which contains an important stage — the definition of a mathematical model that characterizes the connection of the optimization parameter with the main factors. Using such a simplified model allows conclusions accelerating about the significance of various medium components, its qualitative and quantitative composition [7]. The mathematical method of experiment planning allows us to reasonably approach the nutrient medium constructing, making its more economical and technological.

The nutrient medium for cultivation was optimized by the composition of sources of carbon and nitrogen feed, as well as on the content of micro elements. We used the cabbage broth as the basis for preparing the nutrient medium. Cabbage is a vegetable raw material with a unique composition: sugar, pectin, starch, fiber, proteins, pantothenic acid, tartronic acid, carotene, vitamins (C, P, B, PP, K, D and U), micro- and macroelements (K, Na, Ca, Mg, Fe, P, S, Cl, also Co, F,I, Mo, Cu, Zn, Si). To increase the yield of heat-resistant spores and the amount of endotoxin, the nutrient medium was enriched with corn extract, amino acids and mineral salts (Mg, Mn, diammonium phosphate).

Using mathematical method of experiment planning, the nutrient medium was optimized for the cultivation of a new technological strain *B. thuringiensis 87/3* in the conditions of biolabs low tonnage production. The nutrient medium optimization was carried out in the alternation of the maximum and minimum values contents of the media components [8–10]. Regression analysis of experimental results is reflected in Table 2.

Table 2. **Regression analysis of experimental results**

$R^2 = 0.98081$ — regression model determination coefficient of the experimental data						
Coefficients of regression (factors)	**Regression coefficients**	**Standard error**	**Student's coefficient**	**Level of significance p.**	**Trust interval — 95%**	**Trust interval — 95%**
b0	1.006898	0.096511	10.4330	0.000015	0.805579	1.208217
X1	0.092667	0.017812	5.2025	0.000043	0.055511	0.129822
$(X1)^2$	–0.002444	0.000881	–2.7731	0.011733	–0.004283	–0.000606
X2	1.667778	0.067323	24.7727	0.000028	1.527344	1.808212
$(X2)^2$	–0.494444	0.022037	–22.4373	0.000011	–0.540412	–0.448477
X3	0.161000	0.006732	23.9145	0.000034	0.146957	0.175043
$(X3)^2$	–0.005494	0.000220	–24.9331	0.000016	–0.005954	–0.005035

Analyzing the table 2 data it is possible to conclude that all the included factors are statistically significant, as evidenced by their level of significance, a model describing the development of bacteria *Bacillus thuringiensis 87/3* using the studied components. Substitute the table data in the general form of the regression equation:

To assess the adequacy of this model, a dispersion analysis of experimental data and a Fisher's criterion evaluation were performed. The dispersion analysis implementation is reflected in Table 3.

Table 3 shows that the mathematical model included factors (1) adequately describe the investigated process of optimizing the composition of nutrient medium, since the significance level p for each factor is below the permissible level.

The response surfaces to the bacterial colonies development by titration of the investigated process with the reflection of the values factors and the scale of desirability are presented in Fig. 1. Levels of function response to the desirability scale are shown in Fig. 2. Visually analyzing the graphics data, it is possible to clearly state that the optimal composition of the nutrient medium is present in the studied ranges of values factors X1, X2, X3. Implementation of the nutrient medium optimization, according to the developed model, is possible with the help of the desirability function [8]. To determine the nutrient medium optimal composition maximum interval of the investigated response is formed. This takes into account the response surface (Fig. 1) and its maximum levels, set the zero level to $4.11 \cdot 10^9$ cells/ml, and a maximum of $4.41 \cdot 10^9$ cells/ml. Under these conditions it is possible to find the required maximal response values to the desirability function. Implementation of the determination procedure optimization is presented in Fig. 3.

Fig. 3 shows that the optimal components variant is at the intersection of the maximum value of the desirability function in the specified interval of each factor. In this case, rational limits and corresponding optimal values factors that make up the *B. thuringiensis* nutrient medium are determined. For corn extract, this is the interval [8.75 ... 11.25 g/l], and the optimum is 10 g/l; for diamonium phosphate is the interval [1.4 ... 2.0 g/l], and an optimum is 1.5 g/l; for glucose it is the interval [12.5 ... 17.5], and the optimum — 15 g /l.

Table 3. **Dispersion analysis of experimental results**

Factors	**Median deviation**	**Degree of freedom**	**Dispersion**	**Fisher criterion**	**Trust interval p**
X1	0.884830	2	0.442415	218.0776	0.000010
X2	2.079207	2	1.039604	512.4473	0.000035
X3	1.837785	2	0.918893	452.9457	0.000019

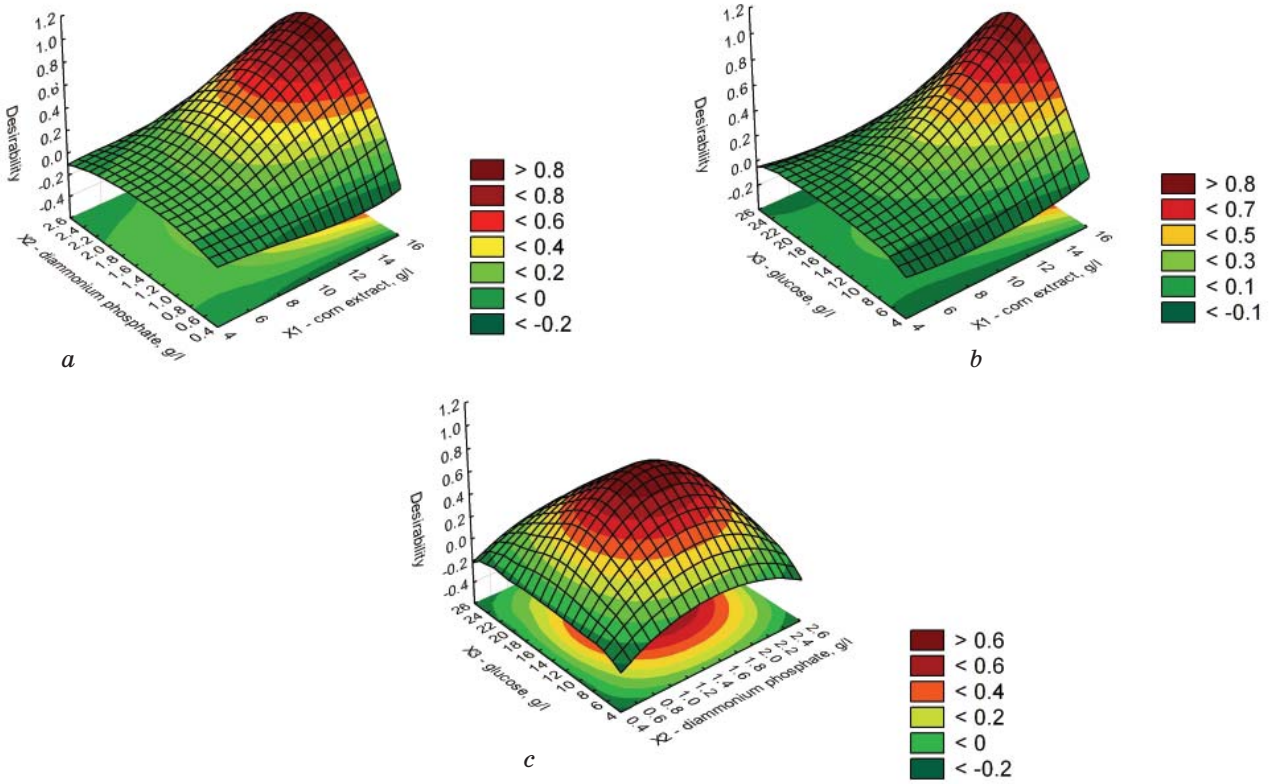

Fig. 1. **Schedule of surfaces response on the desirability scale of the investigated process:** *a* — Y_d(X1, X2); *b* — Y_d(X1, X3); *c* — Y_d(X2, X3)

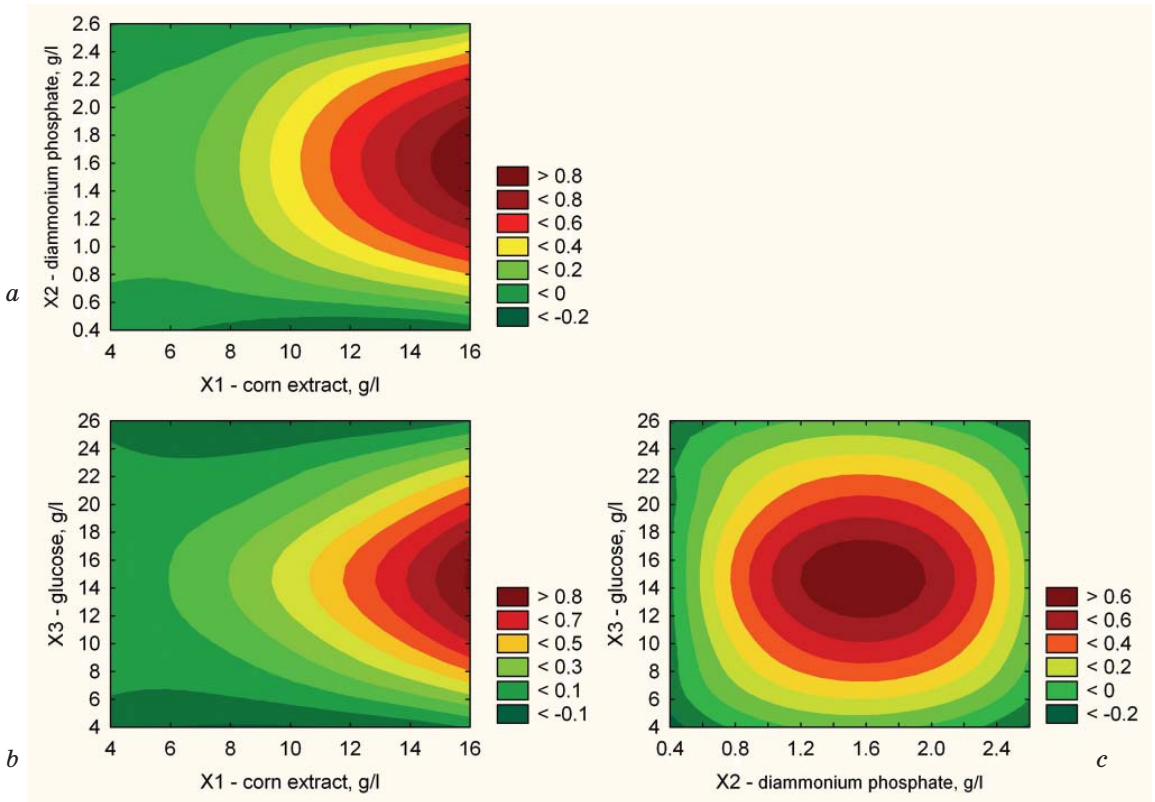

Fig. 2. **Graph of the investigated process response level:** *a* — Y_d(X1, X2) = const; *b* — Y_d(X1, X3) = const; *c* — Y_d(X2, X3) = const

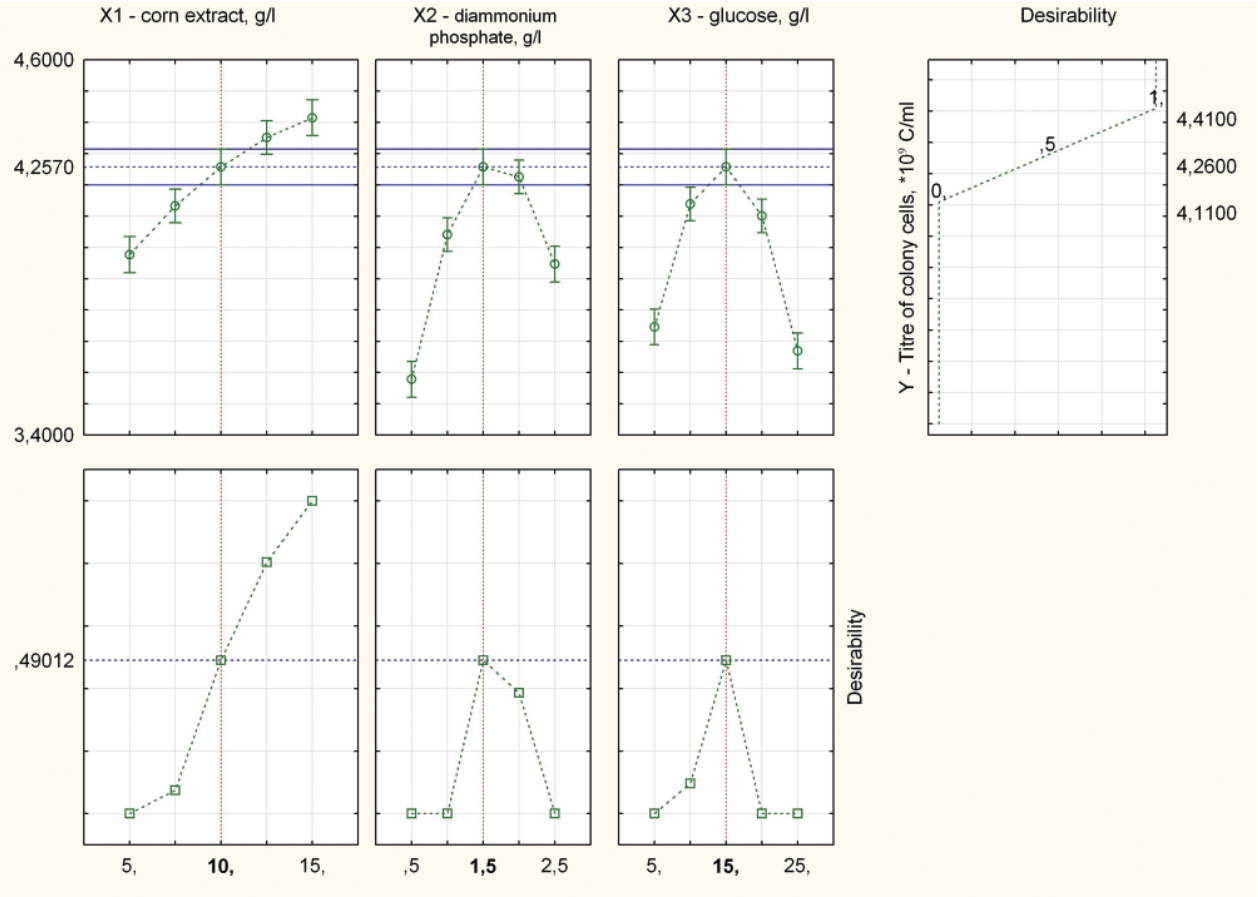

Fig. 3. **Graphic representation of the nutrient medium optimal composition finding procedure**

Using the method of mathematical experiment planning the nutrient medium was optimized for the cultivation of a new technological strain *B. thuringiensis* 87/3. The results of the studies indicate that optimized medium is intended in laboratory conditions and provides the ability to obtain a high yield of viable cells in 24 hours of cultivation (the titre of the metabolic spore-crystalline complex is up to 4.4 billion/ml of culture liquids). The nutrient medium proposed composition is much cheaper than laboratory medium, which are widely used for cultivation microorganisms of this species and can be recommended for using in laboratory and production conditions.

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МАТЕМАТИЧНЕ МОДЕЛЮВАННЯ ОПТИМІЗАЦІЇ СКЛАДУ ЖИВИЛЬНОГО СЕРЕДОВИЩА ДЛЯ КУЛЬТИВУВАННЯ ШТАМУ ЕНТОМОПАТОГЕННИХ БАКТЕРІЙ *Bacillus thuringiensis* 87/3

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Метою дослідження було розробити математичне моделювання оптимального складу рідкого живильного середовища для бактеріального штаму *B. thuringiensis* 87/3, що є оптимальним для отримання біологічно активних компонентів. У роботі використовували нові ентомопатогенні бактерії штаму *B. thuringiensis var. thuringiensis (Bt H1)* № 87/3, селекціоновані *in vitro*, що їх виділено з личинок природних популяцій листогризних комах *Leptinotarsa decemlineata* Say (L_4). За допомогою повнофакторного експерименту за планом Бокса-Бенкіна (3^3) оптимізовано живильне середовище для культивування штаму *Bacillus thuringiensis* 87/3. Визначено оптимальні концентрації в середовищі джерел вуглецю (15 г/л глюкози), азоту (10 г/л кукурудзяного екстракту) та фосфоровмісних неорганічних солей (1,5 г/л діамонію фосфату). Під час культивування цього штаму в періодичних умовах за 30 °С кількість життєздатних клітин досягала $4{,}4 \cdot 10^9$ КУО/мл. Оптимізоване середовище можна рекомендувати для культивування штаму *Bacillus thuringiensis* 87/3 у промислових умовах.

Ключові слова: оптимізація, культивування, *B. thuringiensis* var. *thuringiensis*, живильне середовище.

МАТЕМАТИЧЕСКОЕ МОДЕЛИРОВАНИЕ ОПТИМИЗАЦИИ СОСТАВА ПИТАТЕЛЬНОЙ СРЕДЫ ДЛЯ КУЛЬТИВИРОВАНИЯ ШТАММА ЭНТОМОПАТОГЕННЫХ БАКТЕРИЙ *Bacillus thuringiensis* 87/3

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Целью исследования было разработать математическое моделирование оптимального состава жидкой питательной среды для бактериального штамма *B. thuringiensis* 87/3, оптимальный для получения биологически активных компонентов. В работе использовали новые энтомопатогенные бактерии штамма *B. thuringiensis var. thuringiensis (Bt H1)* № 87/3, селекционированные *in vitro*, выделенные из личинок природных популяций листогрызущих насекомых *Leptinotarsa decemlineata* Say (L_4). С помощью полнофакторного эксперимента по плану Бокса-Бенкина (3^3) оптимизирована питательная среда для культивирования штамма *Bacillus thuringiensis* 87/3. Определены оптимальные концентрации в среде источников углерода (15г/л глюкозы), азота (10 г/л кукурузного экстракта) и фосфорсодержащих неорганических солей (1,5 г/л диаммония фосфата). При культивировании данного штамма в периодических условиях при 30 °С количество жизнеспособных клеток достигала $4{,}4 \cdot 10^9$ КОЕ/мл. Оптимизированную среду можно рекомендовать для культивирования штамма *Bacillus thuringiensis* 87/3 в промышленных условиях.

Ключевые слова: оптимизация, культивирование, *B. thuringiensis* var. *thuringiensis*, питательная среда.