Global Journal of Science Frontier Research: D
Agriculture and Veterinary
Volume 18 Issue 8 Version 1.0 Year 2018
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4626 & Print ISSN: 0975-5896

# Optimization of Nutrient Medium Composition for Entomopathogens Bacillus Thuringiensis Cultivation

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*GJSFR-D Classification: FOR Code: 050299*


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# Optimization of Nutrient Medium Composition for Entomopathogens Bacillus Thuringiensis Cultivation

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## I. Introduction

Microbiological method of protecting plants using biotic origin components and metabolic preparations based on living cultures of microorganisms is an important scientific and practical dimension of biotechnology in agricultural science.The biological pesticide is one of the most promising alternatives over conventional chemical pesticides, which offers less or no harm to the environments and biota. A wide 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 that belong to the species Bacillus thuringiensis (Bt)[1,2]. There are more than 80 serotypes of Bt, selectively specific to the definite groups of host insects belonging to the order Lepidoptera, Diptera, Coleoptera, Hymenoptera, Orthoptera, Hemiptera, Isoptera, Mallophaga, 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 crucial 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.

Application of the traditional methods of protection such as crop rotation, selection of resistant to disease breeds, spraying pesticides is not sufficient to control the contamination of plants. It is possible to reduce using of chemicals, which can serve as an alternative biotechnological application of antagonistic microorganisms. Thus, the importance of biological control of pathogens increases. This also conduces the necessity of pesticide load reduction on agrocenosis and searching ecological alternative to chemicals. Therefore, studying the g. Bacillus new strains multifunctional properties are relevant to expanding the efficient use of bio-agents in agricultural technologies.

*Objectives*: To develop an optimal liquid medium composition for bacteria strain B.thuringiensis $87/_3$, which are the most favorable for the production of biologically active components.

## II. Materials and Methods

The research was conducted by the National University of Life and Environmental Sciences of Ukraine laboratory, 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 by a priori information analysis.

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Obtaining pure cultures, preparation serial dilutions of bacterial suspensions, cultivation on liquid and agar nutrient media conducted by the classical scientific and methodological works in microbiology. During the development, optimal process conditions of cultivation Bt strains determined the following parameters: producing pure capacity culture through boundary dilutions, the rate of formation entomocidal metabolites (spore-crystal complex) the percentage of biotest deaths - larvae of Leptinotarsa decemlineata Say. $L_{1-2}$ when infected culture liquid at the dilution of 1:1; 1:10; without dilution. The biological activity of liquid formulations Bt strains evaluated in model experiments on intact and contact populations of Leptinotarsa decemlineata Say. $L_{1-4}$ three replications (25 larvae in each).The number of dead beetles accounts for 5,7,10-day experiment according to Abbot formula:

$$A = \frac{M_o - M_c}{100 - M_c} \times 100$$ , where

A – entomocidal activity, (%); $M_0$ – the percentage of dead larvae in experiment; $M_c$ – the percentage of dead larvae in control. Death in control will not exceed 15.0 %.

The task of the 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. 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 an 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 15g/l.

Cultivation was carried out in Erlenmeyer's flasks on biotechnology shaker with term platform (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 take into account three factors and their three levels. In this case, the number of experiments that must be carried out can be calculated by the formula:

$$N_{å} = \left(N_{ô}\right)^{ð} \quad (1)$$

Where $N_{ô}$ - the number of experiments, p – the number of levels factors.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. To reduce the impact on the results of the response, the experiments were carried out in a random sequence. The processing of the experiment results began with regression analysis: the model was built and unknown coefficients were determined:

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

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:

$$b_0 = \sum_{i=1}^{N} Y_i \Big/ N;\ b_j = \sum_{U=1}^{N} Y_i X_{ji} \Big/ N;\ b_{j^2} = \sum_{i=1}^{N} Y_i \left(X_{ji}\right)^2 \Big/ N \quad (3)$$

Where, $\sigma^2_{ç.ð.}$, $\sigma^2_Y$-dispersion of residues regression, response; $Y_i$, $\overline{Y}$, $\hat{Y}_i$ - actual, average, estimated value of response.

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

$$S_{b_j r} = \sqrt{\frac{\sum_{i=1}^{N} \left(Y_i - \hat{Y}_i\right)^2}{\sum_{i=1}^{N} \left(X_{ij} - \overline{X}_j\right)^2} \cdot \frac{1}{n-2}} \quad (4)$$

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:

$$\left| t_{\alpha, f} \right| = \left| \frac{b_{j^r}}{S b_{j^r}} \right| \geq t_{\alpha/2, f.\text{òàá}} \qquad (5)$$

Where $b_{j^r}$ - estimated regression coefficients, $\alpha$ - 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:

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$$\Delta_{j^r} = t_{\alpha, f.\text{òàá}} \cdot S b_{j^r} \qquad (6)$$

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

$$b_{j^r} - \Delta_{j^r} \leq b_{j^r} \leq b_{j^r} + \Delta_{j^r} \qquad (7)$$

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:

$$F = \frac{\sigma^2_{\tilde{O}}}{\sigma^2_Y} = \frac{R^2}{1-R^2} \cdot \frac{f_2}{f_1} \geq F_{\alpha, f.\text{òàáë}} \qquad (8)$$

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

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

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

## III. 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 trial, 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 were 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 microelements. 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 macro –elements (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 the 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 [8,9]. Regression analysis of experimental results is shown in table 2.

*Table 1:* 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, (p<0,05) | Trust interval - 95% | Trust interval -95% |
| $b_0$ | 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 1 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:

$$Y = (b_0 + b_1 \cdot X1 + b_2 \cdot (X1)^2 + b_3 \cdot X2 + b_4 \cdot (X2)^2 + b_5 \cdot X3 + b_6 \cdot (X3)^2) \cdot 10^9 =$$
$$= (1{,}006898 + 0{,}092667 \cdot X1 - 0{,}002444 \cdot (X1)^2 + 1{,}66778 \cdot X2 -$$
$$- 0{,}49444 \cdot (X2)^2 + 0{,}161 \cdot X3 - 0{,}005494 \cdot (X3)^2) \cdot 10^9, \text{КУО/мл} \quad (9)$$

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 2.

*Table 2:* Dispersion analysis of experimental results

| Factors | Median deviation | Degree of freedom | Dispersion | Fisher criterion | Trust interval p, (p<0,05) |
|---|---|---|---|---|---|
| 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 |

Table 2 shows that the mathematical model included factors (1) adequately describe the investigated process of optimizing the composition of the nutrient medium, since the significance level for each factor is below the permissible rate.

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 figure 1. Levels of function response to the desirability scale are shown in figure 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 [10]. 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 rate, set the zero level to $4.11 \times 10^9$ cells/ml, and a maximum of $4.41 \times 10^9$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 figure 3.

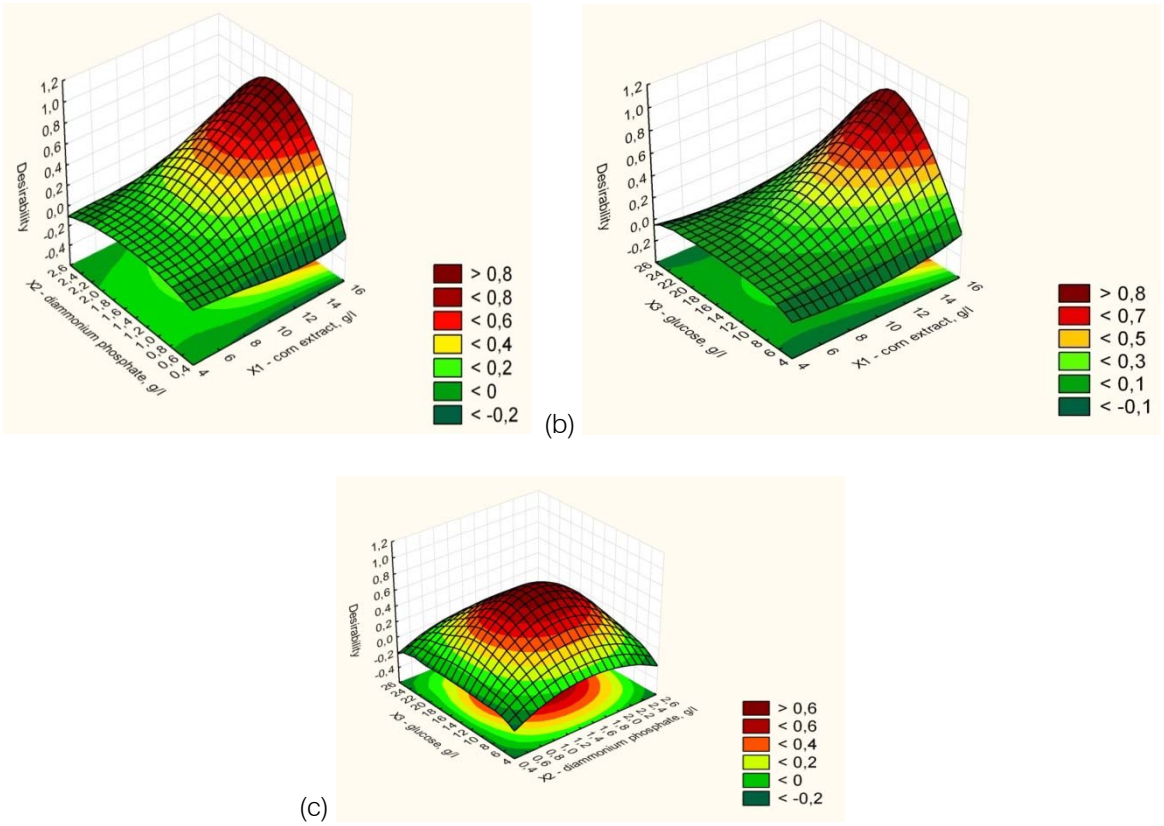


*Fig. 1:* Schedule of surfaces response on the desirability scale of the investigated process: а) – $Y_d(X1, X2)$; б) $Y_d(X1, X3)$; в) $Y_d(X2, X3)$

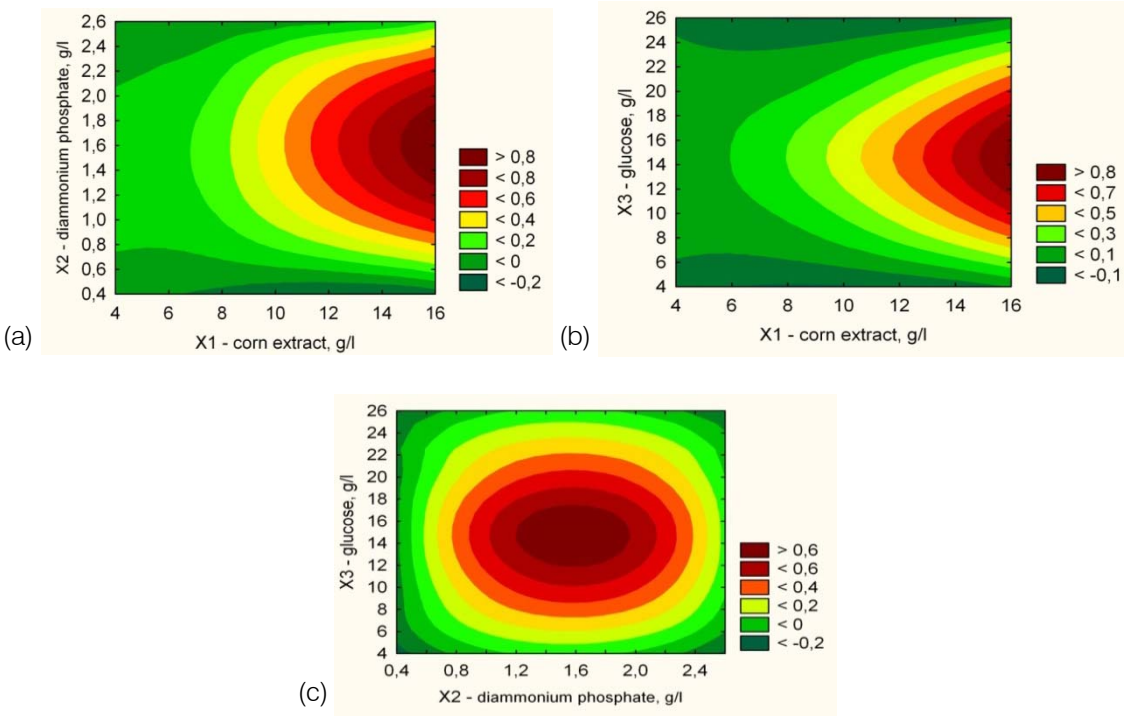


*Fig. 2:* Graph of the investigated process response level: а) – $Y_d(X1, X2)$ = const; б) $Y_d(X1, X3)$ = const; в) $Y_d(X2, X3)$ = const

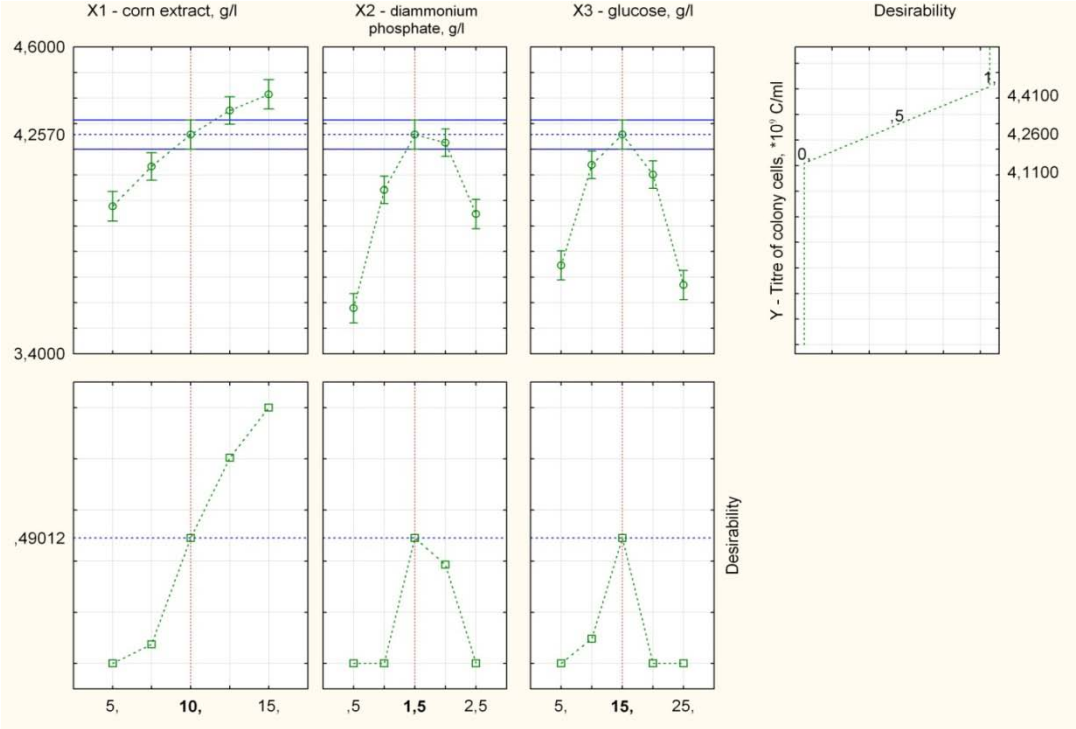


*Fig. 3:* Graphic representation of the nutrient medium optimal composition finding procedure

Figure 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 diammonium 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 - 15g /l.

Expressive maximum death larvae Leptinotarsa decemlineata Say younger generation ($L_{1-2}$) on the 10th day of the experiment is detected on infection load of 1:1. Thus, on the 10th day of the experiment there were about 96,0–99,0% death larvae. When infected of bacterial suspensions Bt with lower spores titer (2,0–2,3 billion spores/ml) on meat infusion agar and LB media options entomocidal variables that do not exceed 89,0% are obtained. The evaluation of the Bt liquid formulations antiphidant effect, which manifests itself through the taste buds of the colorado potato beetle in the field experiments showed that larvae consume only a small part of the leaf surface of the plants (compared to control noninfected ones), noticeably lag in growth and development. In the first days of experience the process of reducing number of pest larvae occurred slowly, but the plants were not damaged, which is due to the antiphidant effect. In the following days of experience there was a metamorphosis violation and death of individuals of the next phase of development.

## IV. Conclusion and Recommendations

The results of the studies indicate that optimized medium for B. thuringiensis $87/_3$ strain cultivation is intended in laboratory conditions and provides the ability to obtain a high yield of viable cells in 24 hours of cultivation (the titer 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 is widely used for cultivation microorganisms of this species and can be recommended for using in laboratory and production conditions. Research of accumulation Bt culture biomass in different medium and liquid preparations functional capacity (biotesting on insecticidal, death Leptinotarsa decemlineata Say. $L_{1-2}$ more than 90.0%) provides an opportunity to deepen the scientific theoretical knowledge and practical approaches in the field of biotechnology and analysis of cultures in gradient media, which compositionally close to natural.

## References Références Referencias

1. Kandyibin N.V. Mikrobiokontrol chislennosti nasekomyih i ego dominanta Bacillus thuringiensis. – M.: SPb, Pushkin: Nauchnoe izdanie «Innovatsionnyiy tsentr zaschityi rasteniy», 2009. – 252 c.
2. Lamenha C. Bacillus sphaericus and Bacillus thuringiensis to Insect Control. Process

Development of Small Scale Production to Pilot-Plant-Fermenters Federal University of Pernambuco Brasil. 2012; 613-627.
3. Roh J.Y., Choi J.Y., Li M.S., et all. Bacillus thuringiensis as a specific, safe and effective tool for insect pest control. J. Mol. Biol. 2007; 17:547-559.
4. Biryukov V.V., Kantere V.M. Optimizatsiya periodicheskih protsessov mikrobiologicheskogo sinteza.– M.: Nauka, 1985. – 296 c.
5. Avignone-Rossa C., Arcas J., Mignone C. Bacillus thuringiensis growth, sporulation and δ-endotoxin production in oxygen limited and non-limited cultures. World Journal of Microbiology and Biotechnology. 1992; 8: 301-304.
6. Harrington E. C. The Desirability Function. Industrial Quality Control,1965. April. P. 494–498.
7. Mason, R., R. Gunst and J. Hess. Statistical Design and Analysis of Experiments With Applications to Engineering and Science. John Wiley &Sons, Inc., 2003; 585-586.
8. Anderson RKI, Jayaraman K. Impact of balanced substrate flux on the metabolic process employing fuzzy logic during the cultivation of Bacillus thuringiensis var. Galleriae. World Journal Microbiology and Biotechnology. 2005; 21:127–133. doi: 10.1007/s11274-004-3043-1.
9. Kraemer-Schafhalter A., Moser A. Kinetic study of Bacillus thuringiensis var. israelensis in lab-scale process. Bioproc Eng.1996;14:139–144.
10. Popovic M., Liu W., Iannotti E.L., Bajpai R.K. A mathematical model for vegetative growth of Bacillus thuriniensis .Eng Life Sci.2001; 2:85–90.