Development of laboratory regulations elements for the pesticide production of based on the entomopathogenic strain *Bacillus thuringiensis* 0271

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Abstract. The production of new entomocidal biologics based on *Bacillus thuringiensis* bacteria does not lose its relevance. In this regard, studies have been conducted on the individual elements development of the regulations for the production of biological insecticide. It has been shown that the liquid formulation obtained on nutrient media SG, PS and No. 9, without stabilizers, retains a spore titer of at least 1.2 billion/ml for 6 months. The culture of the strain *B. thuringiensis* 0271 obtained on nutrient media No. 7 and No. 9 is able to withstand heat treatment no higher than 70°C and no longer than 30 minutes. Simulation of fermentation conditions of strain *B. thuringiensis* 0271 made it possible to predict that the maximum spore titer can be achieved by maintaining a gas pressure of at least 0.7 air volume per medium volume per minute, increasing the temperature at the end of cultivation to 32°C, and a pH in the stationary phase of at least 8.0.

1 Introduction

The issue of obtaining new promising biological products effective against leaf-eating pests does not lose its relevance. The market of modern biologic insecticides is dominated by one based on entomopathogenic bacteria Bacillus *thuringiensis*. Industrial preparations of this type are effective against more than 400 species of insects from the families Lepidoptera, Diptera, Hymenoptera, Coleoptera, Orthoptera [1-3].

There are several reasons for the development of interest in *B. thuringiensis*. This group of bacteria has a specific effect on insect pests, the use of *B. thuringiensis* does not cause persistent contamination of soil, leaves and air with entomopathogenic bacteria. In addition, *B. thuringiensis* is a natural component of biocenoses. It is safe for entomophages and is not pathogenic to mammals and humans. It is the widespread use of *B. thuringiensis* in the practice of pest control, their selective action, safety in relation to the components of agrocenosis and human health necessitates the search and study of new strains. In addition,

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strains with a high entomopathogenic and technological properties are used both for the development of new-generation biologics and as analogues to producing strains of existing ones. *B. thuringiensis* is a gram-positive, spore-forming bacteria capable of forming parasporal crystalline inclusions of a protein nature, Cry and Cyt toxins during sporulation, which are mainly determine their insecticidal activity [4].

The main part of biological insecticides is developed on the basis of *B. thuringiensis* var. *thuringiensis* (Bitoxibacillin, Bitoxin, etc.), which are used against a complex of leaf-eating insects on vegetable, fruit and berry crops, in garden and park plantations and in forests.

Increased interest in microbial insecticides based on *B. thuringiensis* has led to an active search, study of biological properties and selection of microorganisms promising for pest control [5]. No less relevant are the issues of studying the technological characteristics of entomopathogens, developing regulation treatment for the production of biological insecticides based on it. However, there are a number of reasons that do not ensure the success of research. Such reasons include the lack of research on their unstable effect when used and the issue of developing formulations [6].

Modeling processes in modern biotechnology of microorganisms can significantly reduce the amount of time that must be spent on nutrient media optimizing, as well as the parameters of bacterial culture cultivation [7]. Microorganisms are usually cultivated on dense or liquid nutrient media. Under fermenter conditions, parameters such as pH, temperature, quantitative and qualitative composition of incoming and outgoing gases are available for external cultivation. Individual sensors or mass spectrometers are usually used to account for these indicators. Fermenters are equipped with pumps for supplying media, defoamers. Analog units or desktop computers are widely used as automatic regulators. Laboratory fermenters often have a volume of 100 ml to 10 liters. Biotechnological processes of bacterial growth and development are the germination of bacterial cells and spores, as well as the transformation of the substrate into a building material for the cell [8, 9].

In most cases, the attention of researchers is focused on the rate of biomass growth and the consumption of substrates, among which a special place is occupied by the source of energy for growth, oxygen consumption, gas release, the formation of spores and organic metabolic products [10, 11].

2 Materials and Methods

Research work was accomplished at the Molecular Genetic Proteomic and Bioinformatic Laboratory Agricultural Microbiology Department of Research Institute for Agriculture of Crimea. The entomopathogenic strain *B. thuringiensis* 0271 from the Crimean collection of microorganisms (http://www.ckp-rf.ru//507484) for improve the crop yields were used as a material for research. The strain were isolated from the natural populations of insects of Crimean peninsula in the Agricultural Microbiology Department of Research Institute for Agriculture of Crimea.

The number of microbial cells in the preparations and the titer of the spores were determined by the method of sequential dilutions with seeding in Petri dishes on appropriate nutrient media [12]. The study of cultural properties was carried out according to generally accepted methods [13].

The planning of the multifactorial experiment was carried out according to V.N. Maksimov and Yu.M. Voznyakovskaya [14, 15]. The manufacturability of the strains was determined by S.D. Pert [16]. Also, MS Excel 2003 application software packages were used to solve general research and special statistical tasks related to optimizing the composition of nutrient media, the selection of additives and stabilizers, cultivation

parameters, and visualization of the data obtained. Visualization of the experimental results was carried out by constructing a heat map of the expression of toxin formation genes in a liquid culture of the strain *B. thuringiensis* 0271, on promising nutrient media.

The cultivation model of the *B. thuringiensis* strain was constructed according to the documentation of Anylogic Personal learning edition 8.9.1.

3 Results

The nutrient medium for cultivation of the *B. thuringiensis* strain 0271 was optimized. To increase the manufacturability of the *B. thuringiensis* 0271 strain, nutrient media based on corn extract, yeast autolysate, molasses, corn and soy flour were studied. All the media used in this work are developed by the Molecular Genetic Proteomic and Bioinformatic Laboratory. Previously, the technological parameters of cultivation were studied during the cultivation of the entomopathogen on technological rockers in flasks with a volume of nutrient medium of 50 ml. It was found that during cultivation in a SG medium containing corn extract and glucose, strain 0271 did not significantly increase the basic technological characteristics (Table 1), but medium No. 7, consisting of corn extract, molasses and soy flour, contributed to a more active vegetative growth of bacteria. In the stationary phase of culture development (after 10-12 hours of cultivation), the CFU titer reached 4,3–4,2·10^8 in 1 ml of liquid, which was 1.4 times higher than the CFU titer in the SG medium. Accordingly, the titer of viable spores of the liquid culture obtained during cultivation reached 2.6–3.4 billion spores in 1 ml of liquid, which was 36-48% higher than the titer of the liquid spore culture of the SG medium.

Table 1. Technological indicators of the development of the strain *B. thuringiensis* 0271 cultivated in liquid nutrient media with a volume of 50 ml

Stages of bacterial development	Nutrient mediumes			
	SG (control)	PS	No. 7	No. 9
	Observation time, h.			
Stationary phase	13-14	14-16	10-12	13-15
Formation of a sporogenic zone	24-26	18-22	16-19	17-20
Mass sporulation	28-31	26-30	22-25	24-28
10% of free spores	36-38	40-45	36-38	46-48
Spores and crystals in a free state	68-72	66-70	46-49	65-69

The culture of the strain *B. thuringiensis* 0271, obtained on a PS medium based on corn extract and molasses, reached the stationary phase for 14-16 hours of development with a CFU titer 3,3–3,8·10^8 in 1 ml of liquid, which did not exceed the CFU titer in the SG medium. However, the titer of viable spores of the liquid culture obtained during cultivation reached 2.1–2.8 billion spores in 1 ml of liquid, which was 23-26% higher than the titer of the liquid spore culture of the SG medium.

The nutrient medium of PS studied in this experiment did not allow for the rapid production of a spore culture, the formation of the spore-crystal complex was completed by 68-72 hours. In the stationary phase for 14-18 hours of cultivation, it did not contribute to the formation of a high CFU titer (no more than 2.5 10^8 in 1 ml of liquid) and a spore titer (no more 1,4–1,7 ·10^9 in 1 ml of liquid).

In the future, the prospect of using medium No. 7 was also shown for the next stage of the regulation – the cultivation of pathogens in bottles with a 400 ml medium volume. A liquid spore culture obtained by cultivating the pathogen in flasks was used as an inoculum.

It was found that in 400 ml of nutrient medium No. 7, bacteria actively passed the entire development cycle and the titer of viable liquid spores reached 2.3–2.7 billion in 1 ml (in the medium of SG – 1.6–2.1 billion spores in 1 ml of liquid).

One of the main aspects of obtaining biopesticide′s formulations is their stability as a result of long-term storage. The longer the spores remain viable in the culture of the strain, the endotoxin crystals and soluble toxins remain unchanged, the longer it is possible to store the insecticide in storage conditions.

The safety of the liquid formulation obtained on the media SG, PS, No. 7 and No. 9 without stabilizing additives was investigated. It was found that the titer of spores in culture without additives decreased evenly for six months and by the end (after 10 months) of the experiment in medium No. 7, the titer of spores was two orders of magnitude lower than at the end of cultivation (Table 2), which made such a culture unsuitable for use as an entomocidal agent. However, after 6 months of storage, the spore culture retained an average spore titer of 12.3 million / ml, which indicates the expediency of it′s storage for six months. By the end of the experiment, the titer of spores of liquid cultures obtained with nutrient media SG, PS and No. 9 did not significantly differ from those at the end of cultivation and amounted to 6.2–7.1 million/ml.

Table 2. Preservation of the liquid formulation based on the strain *B. thuringiensis* 0271

The variant of the experiment	**The spore titer, 10^8**			
	At the end of cultivation 06.05.2022	**Date of microbial research 15.08.2022**	**Date of microbial research 15.11.2022**	**Date of microbial research 02.03.2023**
SG	15.6±1.64	11.13±0.76	7.0±1.54	6.6±1.61
PS	14.16±1.48	10.46±0.56	8.67±1.02	7.19±1.07
No. 7	14.0±0.45	9.67±1.21	12.3±1.18	0.06±0.85
No. 9	15.0±0.88	12.15±1.46	15.2±1.27	6.2±1.36

The heat resistance of spores is an important feature of the *B. thuringiensis* culture, on which not only the possibility of making a pasty, but also a dry preparation form depends. The dry formulation can significantly increase the shelf life and reduce the cost of transporting the insecticide over long distances.

The study of the spores heat resistance of the strain *B. thuringiensis* 0271 was carried out at 60, 70 and 80°C with exposures from 5 to 30 minutes (Table 3). The analysis of the culture liquid after heating at 60°C for 5 minutes, which is 70°C for 20 minutes and 70°C for 30 minutes showed high temperature resistance of the spores obtained on all the studied nutrient media. The titer of the spores did not differ quantitatively from that obtained in the non-processed variants. Considering the change in the number of viable spores in the culture flush from oblique agar (control), a decrease in the titer of viable spores was noted by an order of magnitude, from 10^8 to 10^7 in variants with more stringent heat treatment modes of 70°C 30 min and 8°C 10 min. Heat treatment also contributed to the most active reduction of the titer of spores from 10^9 to 10^7 in cultures obtained on nutrient media No. 7 and No. 9.

Table 3. Heat resistance of *B. thuringiensis* strains 0271 spores

The variant of the experiment	Heat treatment mode and exposure				
	Control (culture without treatment)	**60 °C 5 min**	**70 °C 20 min**	**70 °C 30 min**	**80 °C 10 min**
Nutrient medium No. 7	$11.50 \cdot 10^8$ ± 1.38	$11.60 \cdot 10^8$ ±0.49	$4.19 \cdot 10^8$ ±0.27	$4.64 \cdot 10^6$ ±0.34	$0.1 \cdot 10^8$ ±001.2
Nutrient medium No. 9	$8.83 \cdot 10^8$ ±2.83	$7.77 \cdot 10^8$ ± 0.21	$7.00 \cdot 10^8$ ±0.25	$7.42 \cdot 10^8$ ±0.31	$0.1 \cdot 10^8$± 0.002
Flushing culture from the oblique agar	$7.2 \cdot 10^8$±0.03	$1.41 \cdot 10^8$ ±0.09	$1.20 \cdot 10^8$ ±0.05	$0.15 \cdot 10^8$ ± 0.002	$0.16 \cdot 10^8$ ± 0.002

In the system-dynamic modeling of fermentation conditions of *B. thuringiensis* 0271 strain culture, parameters such as concentrations of organic nitrogen compounds (`concN`) and carbon (`concC`), the amount of oxygen supplied to the flask (`oxigene`), acidity of the nutrient medium (`pH`) and its temperature (`Temp`) were taken into account (Fig. 1). The default value for the `concN` parameter was set as (0.01 : LITER), and the parameters of the `concC` and oxigene as 0.5, a liter was selected as the unit of measurement. The minimum and maximum values from 0.5 to 0.8 were set for the `oxigene` parameter. The `pH` parameter was set in the range from 5.0 to 9.0. The `concN` and `concC` parameters were set in the ranges 30-70 ml and 1.0-2.5 ml. The `Temp` parameter is assigned by default (30.0 : PER_MINUTE) and the value range is 27-32°C. The system-dynamic model used the `MultiplyByHour` tabular function with linear interpolation.

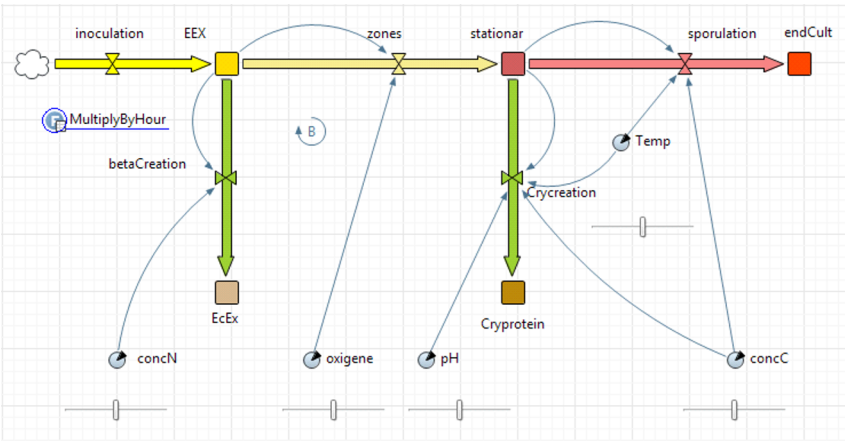

Fig. 1. Scaling model diagram of the obtaining process of liquid spore culture of the strain *B. thuringiensis* 0271 in Anylogic Personal learning edition 8.9.1

The model consisted of three streams corresponding to separate three practically significant stages of culture development - the time of culture development from inoculation to the formation of sporogenic zones in cells (inoculation), the time of subsequent development to the stationary phase (zones) and the time of development to the complete release of spores (sporulation). These streams are limited to the EEX, stationar, and endCult drives, respectively. The inoculation flow is modeled using the

MultiplyByHour(time()) formula, the zones flow using the EEX/oxigene formula, and the sporulation flow using the stationar/Temp/concC formula, taking into account the dependencies between microbiological processes. The model also included the Cry protein and exotoxin formation processes represented by the Crycreation and betaCreation streams limited by Cryprotein and EcEx accumulators. Dependencies in the Crycreation stream were expressed by the formula stationar*pH*concC*Temp, and the betaCreation stream by the formula EEX*concN.

4 Discussion

There is a part of the research for development of a liquid preparative formulation of an entomocidal biological insecticide based on bacteria *B. thuringiensis* 0271. The developed biological product can be recommended against larvae of the Colorado potato beetle on potatoes, tomatoes and eggplants (50 l/ha, two to three treatments against each generation of the pest). The study of the spectrum of action of the drug showed its effectiveness against caterpillars of cabbage scoops on cabbage (50 l/ha, one to three treatments against each generation of the pest). The effectiveness of the drug was taken into account no earlier than 5-7 days after application. The biological efficacy of the drug reaches 79-95%.

The resulting liquid formulation obtained with nutrient media SG, PS and No. 9 retains a spore titer of at least 1.2 billion /ml for 6 months. Thus, media No. 7 and No. 9 are promising for improving the liquid formulation of the entomocidal insecticides and can be recommended for inclusion in the regulations. The resulting mixture is included in the patent application for the invention, which is under review by Rospatent (No. 2022130524; application 11.23.12).

B. thuringiensis 0271 strain culture obtained on nutrient media No. 7 and No. 9 is able to withstand heat treatment no higher than 70°C for no longer than 30 minutes. Since the obtaining culture of *B. thuringiensis* 0271 is heat-resistant, this opens up prospects for obtaining a pasty and dry preparation based on it using modern spray dryers.

Simulation modeling is a chat case of mathematical modeling and allows you to build and implement models that reflect the processes occurring in the system over time. Currently, biotechnology models not only the preparation of bioinsecticides, but also creates tools for analyzing, predicting and modeling data on regulation at the genetic and genomic levels of certain microorganisms. Since the process of cultivating entomopathogenic bacteria in real life takes place over a certain period of time, the simulation method allows the system to reflect it′s behavior in the most appropriate way. The management of such complex systems is often fraught with difficulties, since fermentation occurs in conditions of continuous change in the state of the system, including due to the reaction to the operator's control actions, if the cultivation scheme assumes their presence. During the cultivation of the strain *B. thuringiensis* 0271, after the exponential growth phase, two main entomicidal products (green arrows) are synthesized – a thermostable exotoxin and a crystalline protein thermolabile endotoxin (Cry protein) (Figure 2). The synthesis of the exotokine is significantly influenced by the amount of nitrogen source in the nutrient medium, which in this case is corn extract. The synthesis of endotoxin depends to a greater extent on the amount of oxygen available to the culture and a change in the pH of the nutrient medium in the range of 6.5–8.5. However, both the exotoxin yield and the steady-state release of free spores are influenced by a number of factors. For example, the amount of a carbon source substance that supplies energy to the Krebs cycle, necessary for the synthesis of substances on which cell wall lysis and spore release depend. The second important factor is an increase in temperature in the fermentation flask of the bioreactor from 27 to 30-34 ° C, at which metabolic processes intensify and, accordingly, the yield of the target product increases.

The model was also based on the titer data of a liquid spore culture obtained over time (from 15 to 89 hours of cultivation).

By simulating the fermentation conditions and changing the cultivation conditions in the Anylogic simulation environment, it was possible to predict that the maximum yield of all target products can be achieved under certain theoretically calculated conditions. At optimal concentrations of carbon sources (5 g/l glucose) and nitrogen (50 g / l corn extract), it is advisable to maintain a gas pressure of at least 0.7 air volume per medium volume per minute, increase the temperature at the end of cultivation to 32°C, and the pH in the stationary phase should be at least 8.0.

5 Conclusions

It has been shown that the liquid formulation obtained on nutrient media SG, PS and No. 9, without stabilizers, retains a spore titer of 0271 strain at least 1.2 billion/ml for 6 months. It was revealed that the culture of the strain *B. thuringiensis* 0271 obtained on nutrient media No. 7 and No. 9 is able to withstand heat treatment no higher than 70°C and no longer than 30 minutes. Simulation of fermentation conditions of strain *B. thuringiensis* 0271 made it possible to predict that the maximum yield of all target products can be achieved by maintaining a gas pressure of at least 0.7 air volume per medium volume per minute, increasing the temperature at the end of cultivation to 32°C, and a pH in the stationary phase of at least 8.0.

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