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MODIFIED SEMISYNTHETIC MEDIUM MMBt FOR PRODUCTION OF PREPARATIONS BASED ON *Bacillus thuringiensis*

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Abstract

One of the trends in the biological control of pests is the use of bacteria belonging to the genus *Bacillus* and, first of all, entomopathogenic strains of *Bacillus thuringiensis.* Of great interest to industrial biotechnology are studies related to the search for optimal cultivation conditions that can improve the manufacturability of the production of microbiological preparations and their effectiveness. Previously, the nutrient media for the production of microbiological preparations based on *B. thuringiensis* which include natural organic components have been developed. Nevertheless, during the production of biopreparations based on this bacterium, the foaming of the culture frequently occurs and expensive filters of bioreactors have to be replaced. Also, during the treatment of plants, working solutions containing organic components of the liquid medium can clog the nozzles. This effect complicates the treatment process. In addition, organic cultural media components are not standard and depend on the quality and source origin. In this regard, it is important to carry out the screening of optimal synthetic media that could eliminate these shortcomings. Our study was aimed at selecting the optimal synthetic media and evaluating the effectiveness of the obtained preparation samples in laboratory and field conditions. The objects of study were the cultures of *B. thuringiensis* var. *thuringiensis* 800/15 (BtH_1 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH_{10} 25). The composition of the culture media was as follows: CCY medium — 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM $MnCl_2 \cdot 4H_2O$, 0.05 mM $FeCl_3 \cdot 6H_2O$, 0.05 mM $ZnCl_2$, 0.2 mM $CaCl_2 \cdot 6H_2O$, 13 mM KH_2PO_4, 26 mM K_2HPO_4, 20 mg /l glutamine, 1 g/l casein hydrolysate, 0.4 g/l yeast extract, 0.6 g/l glycerol; MBt medium: 7 g/l casein hydrolyzate, 6.8 g/l KH_2PO_4, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.0022 g/l $MnSO_4 \cdot 4H_2O$, 0.014 g/l $ZnSO_4 \cdot 7H_2O$, 0.02 g/l $Fe_2(SO_4)_3$, 0.18 g/l $CaCl_2 \cdot 4H_2O$; LB medium: 10 g/l trypton, 5 g/l yeast extract, 10 g/l NaCl; modified semi-synthetic medium MMBt (modified MBt): 7 g/l casein hydrolyzate, 6.8 g/l KH_2PO_4, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.0022 g/l $MnSO_4 \cdot 4H_2O$, 0.014 g/l $ZnSO_4 \cdot 7H_2O$, 0.02 g/l $Fe_2(SO_4)_3$, 0.18 g/l $CaCl_2 \cdot 4H_2O$ (25), glucose (1.0 %), Na citrate (2 g/l). Yeast polysaccharide media (YPM) for BtH_1 and BtH_{10} served as a reference. Bt strains were cultivated in 750 ml Erlenmeyer flasks filled with 40-50 ml of medium on a shaker at 220 rpm and 29 °C for 48-72 h until the maturation of culture, accompanied by the formation of spores and crystalline endotoxin. On the basis of BtH_1 800/15 and BtH_{10} 25 strains, batches of liquid preparations were obtained, the effectiveness of which was evaluated in 2020 and 2021 on potatoes (*Solanum tuberosum* L.) of the Yantar variety in the Far East (Ussuri district of Primorsky Krai) against *Henoseplachna vigintioctomaculata* Motsch and on potatoes of the Emelya variety in the Tambov region against *Leptinotarsa decemleniata* Say. In the experiments, liquid preparations obtained on YPM and MMBt were used, which were used at consumption rates of 15 and 20 l/ha. The biological effectiveness of the preparations was calculated according to the formula W.S. Abbot. The antifungal activity of the preparation BtH$_{10}$ 25, obtained on MMBt and YPM, was determined by the method of agar blocks in vitro in Petri dishes. The control medium was used without the addition of drugs. Fungi *Botrytis cinerea* Pers (strain C-5) and *Bipolaris sorokiniana* (Sacc.) Shoemaker (strain C-20) served as test cultures. The inhibitory activity was calculated according to the W.S. Abbot. Cultivation of BtH$_1$ 800/15 and BtH$_{10}$ 25 strains on different nutrient media showed that on semi-synthetic media MBt and LB CFU titers were 2 times lower than on YPM, while on CCY medium they were 10 times lower. Their activity, determined by the content of exotoxin, was also lower, but on the MBt medium it was slightly inferior to YPM for BtH$_1$ 800/15. Therefore, MBt medium was chosen for further studies, and the composition of this medium was modified by adding glucose (1.0 %) and Na citrate (2 g/l). The resulting MMBt medium made it possible to achieve a significant increase in titers, activity, and the rate of culture development compared to the initial MBt. In 2020 in the Tambov region, the effectiveness of the preparation based on BtH$_1$ 800/15 obtained on YPM was high against the Colorado potato beetle and on the 5th day was 95.3 %, slightly inferior to the chemical standard. In the case of preparation obtained on MMBt, it was slightly lower (83.3 %), but the protective effect lasted longer, and on day 15 the efficiency was 73.7 %. In 2021, the efficacy of BtH$_1$ 800/15 was lower than in 2020. In the preparation obtained on MMBt, it was slightly inferior to the effectiveness of the preparation obtained on YPM, amounting to 75.3 and 67.7 %, respectively, on the 5th day after treatment. The effect of the BtH$_{10}$ 25 preparation obtained on MMBt was weaker than in the variant with BtH$_1$ 800/15 (47.7 % on day 5). In Primorsky Krai, the high efficacy of liquid preparations against *H. vigintioctomaculata* was also noted. In 2020, at a rate of application of the BtH$_1$ 800/15 preparation of 15 l/ha, the effectiveness in the YPM and MMBt variants was 60.5 and 63.9 %, respectively, on day 5. Similar data was obtained in 2021. The inhibitory activity of the BtH$_{10}$ 25 preparation obtained on MM*Bt* was 12 % higher on day 5 than that of the preparation obtained on YPM, and was 72.3 and 60.8 % for *B. sorokiniana* and 78.9 and 67.4 % for *B. cinerea*. On day 10, this trend persisted, but for the preparation produced on YPM, a decrease in the inhibition of the growth of *B. sorokiniana* and *B. cinerea* colonies, respectively, to 57.3 and 44.3 % was noted. Thus, preparations based on *Bacillus thuringiensis* obtained on the MMBt medium were only slightly inferior in terms of effectiveness against pests to preparations obtained on YPM, while their effectiveness against phytopathogens was higher than that of preparations with YPM. The MM*Bt* medium is promising for agricultural biotechnology, since its use reduces the time required for the formation of spores and crystalline protein endotoxin by increasing the growth rate of the *B. thuringiensis* culture. Thus, on the MMBt medium, this process ends after 48 h, and on the YPM medium, after 72 h, which makes it possible to reduce energy consumption.

Keywords: biopreparation, *Bacillus thuringiensis* var. *thuringiensis*, *Bacillus thuringiensis* var. *darmstadiensis*, *Bipolaris sorokiniana*, *Botrytis cinerea*, Colorado potato beetle, potato ladybug, inhibitory activity, cultural medium

Biologicals against plant pests and pathogens are extremaly relevant for modern agrobiotechnologies. Optimization of cuture to ensure higher efficiency and manufacturability of microbes-based drugs is of particular importance. Environmentally friendly pest control products that are an alternative to chemical pesticides are in the focus. Microorganisms, primarily entomopathogenic bacteria of the genus *Bacillus* are the most promising for creating insect control agents [1]. *Bacillus thuringiensis* is the basis for 90% of biopesticides used commercially to control pests [2]. These drugs are active against insects of the orders *Lepidoptera*, *Diptera*, *Coleoptera*, *Hymenoptera* and nematodes [3, 4]. For biocontrol, three pathovars are most widely used, i.e., A (var. *thuringiensis*, var. *dendrolimus*, var. *galleria*, var. *kurstaki*) mainly against *Lepidoptera*, B (var. *israelensis*) as a producer of larvicidal drugs, and C (var. *tenebrionis*, var. *darmstadiensis*) against beetles [5]. Microbiologicals based on these bacteria are highly toxic to certain groups of insects, safe for humans and have minimal impact on the environment [6-9], reducing the chemical load on the environment.

B. thuringiensis are multifunctional. Along with entomocidal activity, they possess antifungal activity against numerous pathogens that cause dangerous diseases in crops [10-12]. The wide range of *B. thuringiensis* properties is due to a repertoire of genes encoding the synthesis of various protein toxins, minor protein virulence factors, and various low-molecular metabolites [13].

Preparations active against beetles are widely used against the most common and dangerous pests of crops, such as the Colorado potato beetle (*Leptinotarsa decemleniata* Say) and the 28-spot potato ladybug (*Henoseplachna vigintioctomaculata* Motsch). The Colorado potato beetle lives everywhere. It has high fertility, easily adapts to a variety of external conditions and biotic factors. Different stages of the beetle feed throughout the growing season. Crop losses can reach 30-50% or higher, up to complete loss, if the pest is not controlled and its population increases significantly [14].

In the Far East, where about 5% of potatoes in the Russian Federation are produced, along with the Colorado potato beetle, the 28-spotted potato ladybug (*Henoseplachna vigintioctomaculata* Motsch, 1857) causes significant damage to the crop [15]. It can damage 20 to 100% of the leaf surface, resulting in significant yield losses [16].

For the production of *B. thuringiensis* based preparations, nutrient media have been developed, which include corn and soy flour, starch, protein-vitamin concentrate (PVC) and other natural organic ingredients. Some ingredients may be deficient. Good results were obtained when using pea, barley and oat flour as a source of nitrogen [17]. Preparations obtained in these media are highly active, but there are some disadvantages. Foaming of the culture often occurs and as a result expensive filters in fermenters become clogged and require replacement. Difficulties also arise during treatments, since solutions containing organic components of the medium clog the nozzles. In addition, nutrient media of natural organic composition depend on the quality and origin of the raw materials. Hence, a request arose to develop novel media based on relatively inexpensive and standard, mainly synthetic, components to ensure the best growth and biosynthesis of metabolites which in turn would reduce the cost of biopesticide production.

It is known that culture media containing predominantly synthetic components include carbon and nitrogen sources, the derivatives of natural components that increase spore formation and production of protein crystalline endotoxin toxic to insects [18, 19]. Yeast extract [20] and casein hydrolysate as a source of protein [21] are the most commonly used nitrogen sources. Glycerol and glucose predominate as carbon sources [22, 23].

In this work, for the first time, we submit the improved optimal modified semi-synthetic medium MMBt (Modified MBt). The improved medium ensures the production of effective and technologically advanced biologicals based on various serovars of *B. thuringiensis*.

The purpose of the work is to search for optimal nutrient media for *Bacillus thuringiensis* (Bt) to produce and use Bt-based biologials.

Materials and methods. The study involved gram-positive bacteria *B. thuringiensis* var. *thuringiensis* 800/15 (BtH1 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH10 25).

The culture media was as follows. CCY contained 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM $MnCl_2 \cdot 4H_2O$, 0.05 mM $FeCl_3 \cdot 6H_2O$, 0.05 mM $ZnCl_2$, 0.2 mM $CaCl_2 \cdot 6H_2O$, 13 mM KH_2PO_4, 26 mM K_2HPO_4, 20 mg/l glutamine, 1 g/l casein hydrolysate, 0.4 g/l yeast extract, 0.6 g/l glycerol [24]. MWt medium contained 7 g/l casein hydrolyzate, 6.8 g/l KH_2PO_4, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.0022 g/l $MnSO_4 \cdot 4H_2O$, 0.014 g/l $ZnSO_4 \cdot 7H_2O$, 0.02 g/l $Fe_2(SO_4)_3$, 0.18 g/l $CaCl_2 \cdot 4H_2O$ [25]. LB medium was 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl [26]. Modified semi-synthetic medium MMBt was 7 g/l casein hydrolyzate, 6.8 g/l KH_2PO_4, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.0022 g/l $MnSO_4 \cdot 4H_2O$, 0.014 g/l $ZnSO_4 \cdot 7H_2O$, 0.02

g/l $Fe_2(SO_4)_3$, 0.18 g/l $CaCl_2 \cdot 4H_2O$ [25] + glucose (0.5, 1.0 or 2.0%) and Na citrate (2 g/l). Yeast polysaccharide media (YPS) for BtH1 [27] and BtH10 [28] served as a standard.

Bt strains were cultured on a shaker (220 rpm) at 29 °C in 750 ml Erlenmeyer flasks with 40-50 ml of medium for 48-72 h until spores and crystalline endotoxin complete formation. The cell number and exotoxin amount, expressed in LC_{50} for the housefly *Musca domestica* Linn. Larvae were determined as described by S.D. Grishechkina et al. [29]. The experiment was arranged in triplicate.

Based on strains BtH1 800/15 and BtH10 25, batches of liquid preparations were produced in fermenters at the Ecos branch of the All-Russian Research Institute of Agricultural Microbiology (St. Petersburg-Kolpino). The drug field effectiveness was assessed on potato (*Solanum tuberosum* L.) in 2020 and 2021, on Yantar variety in the Far East (Primorsky Territory, Ussuriysk Province) against *H. vigintioctomaculata* and on Emelya variety in the Tambov Province against *L. decemleniata*. Liquid preparations produced using YPM and MMBt were tested. The experiments were carried out in 4 replicates on 10 m^2 plots. No treatment was carried out in the control; the chemical standard against the Colorado potato beetle was the drug Borey (JSC August, Russia; 0.1 l/ha). The pests were counted on days 5, 10 and 15 after treatment. The rates of liquid preparations against Colorado potato beetle was 20 l/ha, against potato ladybugs 15 and 20 l/ha. The number of pests per plant was calculated (4 replicates of 10 plants).

Biological effectiveness (BE) of drugs was calculated according to Abbot'd formula [30]:

$$BE = (C - T)/C \times 100\%,$$

where C is the number of the pest before treatment, T is the number of the pest after treatment.

The antifungal activity of the BtH10 25-based drug produced in MMBt and YPM was determined in Petri dishes by the in vitro agar block method [31]. The preparations (10% concentration) were added to a molten and cooled to 40 °C potato agar. Blocks 0.8 cm in size cut from a 7-day culture of fungi, were placed on the solidified agar. In the control, the medium was without the drugs. Test fungi cultures were *Botrytis cinerea* Pers. (strain C-5) and *Bipolaris sorokiniana* (Sacc.) Shoemaker (strain C-20). There were 4 dishies for each treatment in 4-fold biological repetitions.

Inhibitory activity was calculated by the Abbot's formula [30]: SI (the degree of fungal colony growth inhibition) = $(D_c - D_t)/D_c \times 100\%$, where D_c and D_t are the diameter of the fungal colony in the control and the test, respectively.

The statistical significance of differences was assessed using two-way analysis of variance with Bonferroni correction for multiple comparisons. Means (*M*), standard deviations (±SD) and standard errors of the means (±SEM) were calculated. Data on the effectiveness of drugs against pests were processed by analysis of variance with a 95% confidence interval [32].

Results. BtH1 800/15 and BtH10 25 culturing showed that in semi-synthetic media MBt and LB the titers were 2 times lower than in YPM, and in CCY medium the titers were 10 times lower. The exotoxin concenteration also turned out to be lower, but for BtH1 800/15, in the MBt medium it was inferior to YPM (Table 1), so the MBt was chosen for further studies. According to K.W. Nikkerson et al. [33], glucose and sodium citrate added to the nutrient medium promotes microbial growth and crystal formation. In this regard, we studied the effect of different glucose concentrations (0.5, 1.0 and 2.0%) on the development of *B. thuringiensis* cultures. It was found that at 0.5 and 1.0% glucose the development of the culture ended after 48 hours, but the titers at a 0.5% concentration were lower than at 1.0%. When 2.0% glucose was added, the development of the culture was inhibited and the sporulation ended after 72 hours. Having determined the optimal glucose concentration of 1.0%, we added sodium citrate (2 g/l) to the medium as an additional source of carbon. The titers in the MMBt medium increased in BtH1 800/15 to 1.95 CFU/ml (by 21.8%), in BtH10 25 to 1.8 CFU/ml (by 12%) (see Table 1). Activity rates also increased. In the MMBt medium, culture growth accelerated and sporulation and the crystalline protein endotoxin formation ended after 48 hours, whereas in YPM it ended after 72 hours.

1. Characterization of preparations based on *Bacillus thuringiensis* var. *thuringiensis* 800/15 (BtH$_1$ 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH$_{10}$ 25), gown on different nutrient media (M±SD)

Strain	Medium									
	YPM		MBt		MMBt		CCY		LB	
	1	2	1	2	1	2	1	2	1	2
BtH$_1$ 800/15	3.35±0.10	2.4±0.2	1.60±0.10	3.0±0.1	1.95±0.20	2.8±0.2	0.38±0.10	6.9±0.1	1.30±0.10	6.3±0.2
BtH$_{10}$ 25	3.0±0.2	2.5±0.2	1.5±0.1	4.2±0.1	1.8±0.1	3.4±0.2	0.3±0.1	7.9±0.2	1.1±0.1	6.7±0.2

N o t e. 1 — titer (CFU/ml), 2 — activity by LC$_{50}$ for 2nd instar larvae *Musca domestica*. For a description of the media composition and sample sizes, see the Materials and methods section.

Previously, when culturing *B. thuringiensis* in yeast-polysaccharide media, we selected the doses of inoculum. It was found that doses from 0.2 to 1.0% did not significantly affect the strain productivity [17]. The same range of inoculation (0.2; 0.5; 0.8; 1.0%) turned out to be insufficient for MMBt, and the Bt titers were low. An increased doses of inoculum (4.0; 6.0; 10.0%) showed the best results for 10% inoculum from the nutrient medium volume, which is consistent with the data of V.V. Biryukova [34].

An assessment of the liquid preparation effeciacy against the Colorado potato beetles on the Emelya variety potatoes (the Tambov Province) showed a significant dependence on the number of the pest, which, in turn, depended on weather conditions and averaged 9 bettles per bush in 2020 and 33 bettles per bush in 2021.

2. Bioeffectiveness of liquid preparations based on *Bacillus thuringiensis* var. *thuringiensis* 800/15 (BtH$_1$ 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH$_{10}$ 25) cultures in different nutrient media against Colorado beetle (*Leptinotarsa decemlineata* Say) on potato (*Solanum tuberosum* L.) cv. Emelya (N = 4, n = 10; Tambov Province, 2020-2021)

Treatment	Dosage, l/ha	Average larvae number per plant				Bioeffectiveness, % (M±SD)		
		before treatment	after treatment			day 5	day 10	day 15
			day 5	day 10	day 15			
2020								
BtH$_1$ 800/15 (YPM)	20	8.05	0.38	2.00	3.38	95.3±1.6	74.4±3.1	58.8±4.4
BtH$_1$ 800/15 (MMBt)	20	14.80	2.38	4.98	3.85	83.3±5.0	65.4±8.7	73.7±8.7
Chemical standard								
Borei	0,1	4.70	0.25	0.88	2.05	100	81.4±4.4	56.2±2.9
Control	No tretment	7.10	3.08	2.52	1.30			
LSD$_{05}$						7.7	16.0	14.9
2021								
BtH$_1$800/15 (YPM)	20	36.30	8.96	14.2	8.97	75.3±6.3	60.7±7.7	47.7±3.6
BtH$_1$ 800/15 (MMBt)	20	35.20	11.4	16.2	20.3	67.7±2.1	53.7±5.5	42.5±5.0
BtH$_{10}$ 25 (MMBt)	20	30.45	16.8	18.0	18.5	47.9±3.1	40.8±3.9	39.4±4.9
Chemical standard								
Borei	0,1	35.10	3.75	6.0	11.3	89.3±5.6	82.8±6.1	69.4±4.9
Control	No tretment	25.90	29.4	19.5	10.1			
LSD$_{05}$						12.0	8.9	13.6

N o t e. The use of drugs produced in the MMBt medium did not lead to clogging of the nozzles, unlike the drugs obtained in the YPM.

In 2020, the effectiveness of the BtH1 800/15-based drug produced in the

YPM was high and reached 95.3% on day 5, being practically not inferior to the chemical standard (100%). The effectiveness of the BtH1 800/15-based drug produced in MMBt was slightly lower (83.3%) but the protective effect lasted longer. On day 15, the effectiveness was 73.7% vs. that recorded for the drug produced in the YPM and for the chemical drug Borei, 58.8 and 56.2%, respectively. In 2021, the effectiveness of the drugs was lower compared to 2020 and on day 5 after treatment with $BtH_1$800/15 it differed slightly for MMBt and YPM media (67.7 and 75.3%, respectively). When using the drug BtH10 25 produced in MMBt, the effectiveness was inferior to BtH1 800/15 and on day 5 reachrd 47.9%. In all variants, the effectiveness decreased on day 15 (Table 2).

3. Bioeffectiveness of liquid preparations based on *Bacillus thuringiensis* var. *thuringiensis* 800/15 (BtH_1 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH_{10} 25) cultures in different nutrient media against 28-spotted potato ladybird (*Henoseplachna vigintioctomaculata* Motsch, 1857) on potato (*Solanum tuberosum* L.) cv. Yantar (N = 4, n = 10; Primorsky Krai, Ussuriysk District, 2020-2021)

Treatment	Dosage, l/ha	Average larvae number per plant				Bioeffectiveness, % (M±SD)		
		before treatment	after treatment					
			day 5	day 10	day 15	day 5	day 10	day 15
			2020					
BtH_1 800/15 (YPM)	15	2.7	1.2	0.6	0.2	60.5±7.0	79.5±5.0	86.4±4.9
BtH_1 800/15 (MMBt)	20	3.2	0.9	0.6	0.2	70.5±5.4	84.3±7.1	90.3±3.8
BtH_1 800/15 (MMBt)	15	3.0	1.2	0.5	0.4	63.9±6.7	78.5±4.1	76.2±5.6
Control	No tretment	3.9	4.3	2.8	2.1			
LSD_{05}						9.4	15.5	13.2
			2021					
BtH_1 800/15 (YPM)	15	4.3	1.8	1.0	1.0	76.8±6.4	79.2±4.1	81.0±5.2
BtH_1 800/15 (MMBt)	15	5.7	2.3	1.2	1.2	78.5±5.7	80.4±3.6	82.7±6.9
BtH_{10} 25 (YPM)	15	6.5	3.5	1.5	0.7	72.1±4.9	76.8±5.9	89.6±6.7
BtH_{10}25 (MMBt)	15	9.3	3.0	1.5	1.4	83.0±4.0	86.2±6.0	86.0±5.7
Control	No tretment	3.9	7.9	5.2	4.9			
LSD_{05}						7.6	9.7	13.0

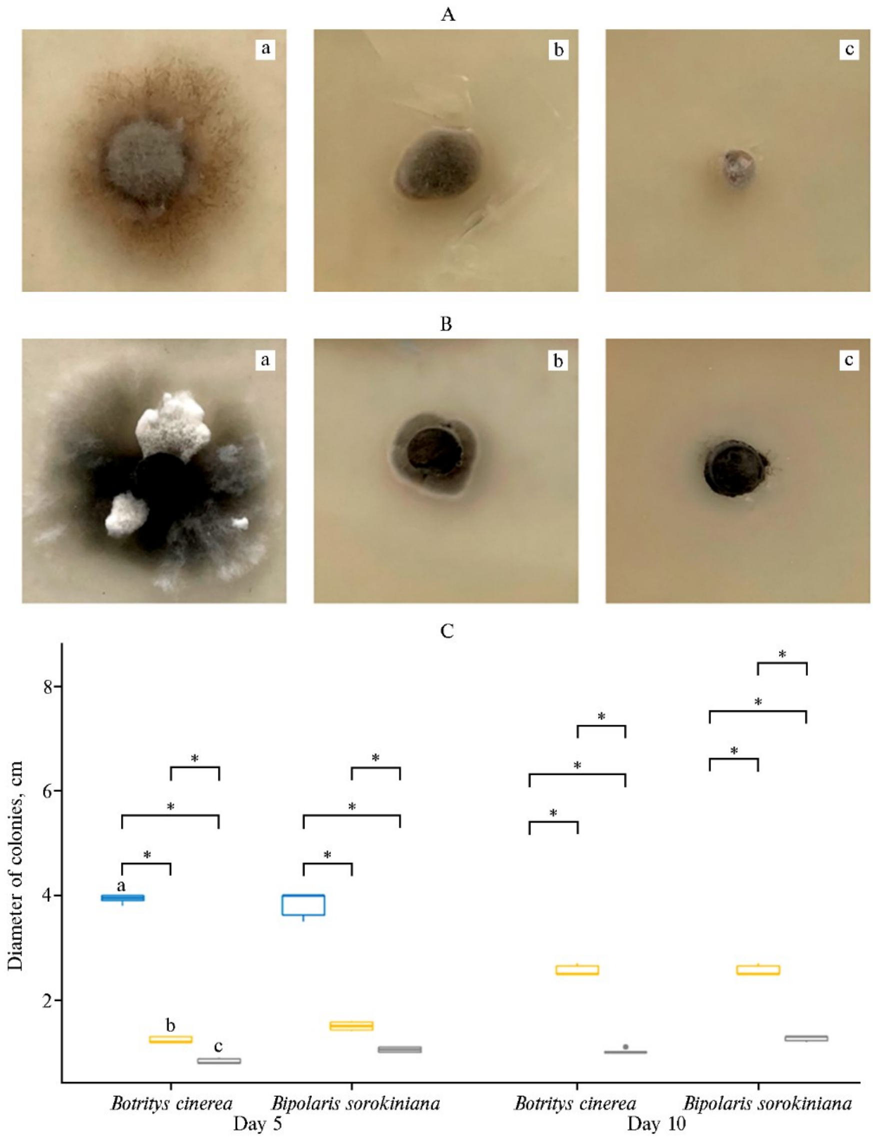
The high effectiveness of liquid preparations produced in different media was also shown when treating potato plantings against the 28-spotted potato ladybug in the Primorsky Territory. In 2020, with the BtH1 800/15 application rate of 15 l/ha the effectiveness for YPS and MMBt media on day 5 was 60.5 and 63.9%, respectively. For MMBt, an increase in the application rate to 20 l/ha increased the efficiency to 70.5%. Similar data we obtained in 2021 (Table 3).

In 2021, along with the drug BtH1800/15, the effectiveness of BtH1025 was studied. On day 5 after treatment, for the preparation in the MMBt medium, the effectiveness was slightly higher than for YPM, up to 83.0% compared to 72.1%. On day 15, the activity of the drugs increased.

Thus, field trials on potatoes against the Colorado beetle and 28-spotted ladybird showed the advantage of MMBt for production of the biologicals.

On day 5 in in vitro tests with BtH10 25, the inhibitory activity of the MMBt-based liquid preparation was 12% higher than that of the YPM-based, for *B. sorokiniana* 72.3 and 60.8%, respectively, for *B. cinerea* 78.9 and 67.4% (Fig.). On day 10, this trend continued. The growth inhibition of *B. sorokiniana* decreased to 57.3%, *B. cinerea* to 44.3% for YPM preparation while for MMBt, the inhibitory activity was not reduced. In the control, on day 5, *B. sorokiniana* and *B. cinerea* showed approximately the same growth rates but differences occurred on day 10.

It should be noted that the diameter of the *B. sorokiniana* and *B. cinerea* colonies on days 5 and 10 differed ($p < 0.0001$) both in the YPM- and MMBt-based liquid preparations vs. control and betwee each other (see Fig., B).



Growth (day 5) of *Botritys cinerea* Pers. C-5 (A) **and *Bipolaris sorokiniana* (Sacc.) Shoemaker C-20** (B) **and the diameter of colonies (days 5 and 10)** (B) **on potato agar after treatment with a *Bacillus thuringiensis* var. *darmstadiensis* 25 (BtH10 25)-based preparation:** a — control (without treatment), b — culture in the YPS medium, c — culture in the MMBt medium (see the description in the Materials and methods section).

* The differences between the treatments are statistically significant at $p < 0.0001$.

The MMBt medium had a significant advantage over the yeast-polysaccharide media widely used for the production of liquid forms of drugs. Since *Bacillus thuringiensis* is one of the most widely used bacteria in biotechnology [35, 36], optimization of media for their growth is of significant interest. Thus, complex substrates such as molasses, corn extract, corn flour [37)], soy flour, etc. [38] are used as organic ingredients. Note, an important factor in the production of biologicals is the lower cost of culture media, and soybean flour is one of the cheapest components [39]. However, its use often leads to foaming of the culture during preparation production, requires filter replacement, ultimately increasing the product price.

A number of studies have also shown the importance of balancing carbon and nitrogen sources in media to achieve optimal sporulation efficiency in Bt cultures. It was found that the higher concentration of glucose in the medium, the higher is optical density of the culture, while increasing the yeast extract concentration suppresses sporulation [40]. Other nitrogen sources can have either stimulatory or inhibitory effects, depending on the culture growth stage [41]. Moreover, a recent study that provided appearantly the most detailed comparative up-to-date analysis of the influence of nitrogen and carbon balance in the culture medium on Bt sporulation showed that the highest sporulation occurs when the carbon to nitrogen ratio in the medium is 5:1 [42]. It is noteworthy that when bacteria of the genus *Bacillus* are used for the production of enzymes (the vegetative culture stage), increasing the nitrogen concentration, on the contrary, can have a beneficial effect [43]. Thus, the selection of media components to optimize bacterial growth significantly depends not only on the systematic position of the microorganisms, but also on the stage of the life cycle (vegetative or spore). Yeast autolysates traditionally used in Bt culture media delay sporulation. In addition, complex organic components reduce the processability of the resulting media.

So, the modified semi-synthetic medium (MMBt) we developed, which consists of casein hydrolyzate and a standard set of salts supplemented with 1% glucose and 2 g/l Na citrate as carbon sources, eliminates shortcomings discussed hereinabove. MMBt is suitable for the production of *Bacillus thuringiensis*-based liquid preparations and has advantages over yeast-polysaccharide media (YPS). Thus, the use of the MMBt medium can ensure the production of *B. thuringiensis* biologicals that are quite effective and more convenient for use. In MMBt medium, the growth rate accelerates and less time is required for the formation of spores and protein crystalline endotoxin. E.g., in the MMBt medium, the formation ended after 48 hours vs. 72 hours in the YPS medium that reduces energy costs. In field trials, there was no clogging of the equipment nozzles for MMBt-based preparations while the YPS-based preparations require washing of the spraying equipment due to contamination with organic particles. Field trials confirm that *B. thuringiensis* var. *thuringiensis* 800/15 and *B. thuringiensis* var. *darmstadiensis* 25 preparations in MMBt are not inferior to YPS preparations in effectiveness and have a longer insecticidal effect against the Colorado potato beetle (the Tambov Povince) and the 28-spotted potato ladybug (the Far East). The use of the developed medium contributed to enhancing the activity of the drug based on *B. thuringiensis* var. *darmstadiensis* 25 against plant pathogenic fungi. In general, the MMBt is a promising and more technologically advanced alternative to yeast-polysaccharide media, avoiding the use of additional organic components which in some cases can be non-standard and difficult to acquire.

REFERENCES

1. Navon A. *Bacillus thuringiensis* insecticides in crop protection — reality and prospects. *Crop Protection*, 2000, 19(8-10): 669-670 (doi: 10.1016/S0261-2194(00)00089-2).
2. Kalmykova G.V., Gorobey I.M., Osipova G.M. *Biotekhnologiya*, 2016, 4: 12-19 (in Russ.).
3. De Maagd R.A., Bravo A., Crickmore N. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics*, 2001, 17(4): 193-199 (doi: 10.1016/S0168-9525(01)02237-5).
4. Marroquin L.D., Elyassnia D., Griffitts J.S., Feitelson J.S., Aroian R.V. *Bacillus thuringiensis* (*Bt*) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. *Genetics*, 2000, 155(4): 1693-1699 (doi: 10.1093/genetics/155.4.1693).
5. Grishechkina S.D. Mechanism and activity spectrum of microbiological preparation batsikol with phytoprotective action. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2015, 50(5): 685-693 (doi: 10.15389/agrobiology.2015.5.685eng).
6. Schnepf E., Crickmore N., Van Rie J., Lereclus D., Baum J., Feitelson J., Zeigler D.R.,

Dean D.H. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews*, 1998, 62(3): 775-806 (doi: 10.1128/mmbr.62.3.775-806.1998).
7. Siegel J.P. The mammalian safety of *Bacillus thuringiensis* — based insecticides. *Journal of Invertebrate Pathology*, 2001, 77(1): 13-21 (doi: 10.1006/jipa.2000.5000).
8. Raymond B., Federici B.A. In defence of *Bacillus thuringiensis*, the safest and most successful microbial insecticide available to humanity — a response to EFSA. *FEMS Microbiology Ecology*, 2017, 93(7): fix084 (doi: 10.1093/femsec/fix084).
9. Belousova M.E., Malovichko Yu.V., Shikov A.E., Nizhnikov A.A., Antonets K.S. Dissecting the environmental consequences of *Bacillus thuringiensis* application for natural ecosystems. *Toxins*, 2021, 13(5): e355 (doi: 10.3390/toxins13050355).
10. Grishechkina S.D., Smirnov O.V., Kandybin N.V. *Mikologiya i fitopatologiya*, 2002, 36(1): 58-62 (in Russ.).
11. Smirnov O.V., Grishechkina S.D. Polyfunctional activity of *Bacillus thuringiensis* Berliner. *Sel'skokhozyaistvennaya biologiya*, 2011, 3: 123-126.
12. Grishechkina S.D., Ermolova V.P., Kovalenko T.K., Antonets K.S., Belousova M.E., Yakhno V.V., Nizhnikov A.A. Polyfunctional properties of the *Bacillus thuringiensis* **var.** *thuringiensis* industrial strain 800/15. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(3): 494-504 (doi: 10.15389/agrobiology.2019.3.494eng).
13. Malovichko Y.V., Nizhnikov A.A., Antonets K.S. Repertoire of the *Bacillus thuringiensis* virulence factors unrelated to major classes of protein toxins and its role in specificity of host-pathogen interactions. *Toxins*, 2019, 11(6): 347 (doi: 10.3390/toxins11060347).
14. Mordkovichskiy K.Z. *Zashchita i karantin rasteniy*, 2016, 3: 36-38 (in Russ.).
15. Kovalenko T.K., Matsishina N.V. *Chteniya pamyati A.I. Kurentsova* [Readings in memory of A.I. Kurentsov]. Vladivostok, 2015, vyp. KhKhVI: 128-136 (in Russ.).
16. Volkov O.G., Smirnov Yu.V., Kovalenko T.K. *Karantin rasteniy. Nauka i praktika*, 2012, 1(1): 41-44 (in Russ.).
17. Grishechkina S.D., Ermolova V.P. Efficiency of batsikol based on a new strain *Bacillus thuringiensis* var. *darmstadiensis* № 25 for biocontrol of phytophagous pests and phytopathogens. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2015, 50(3): 361-368 (doi: 10.15389/agrobiology.2015.3.361eng).
18. Kamoun F., Zouari F.N., Saagdaoui I., Jaoua S. Improvement of *Bacillus thuringiensis* bacteriocin production through culture conditions optimization. *Preparative Biochemistry & Biotechnology*, 2009, 39(4): 400-412 (doi: 10.1080/10826060903209653).
19. Martínez-Cardeñas J.A., de la Fuente-Salcido N.M., Salcedo-Hernández R., Bideshi D.K., Barboza-Corona J.E. Effects of physical culture parameters on bacteriocin production by Mexican strains of *Bacillus thuringiensis* after cellular induction. *Journal of Industrial Microbiology and Biotechnology*, 2012, 39(1): 183-189 (doi: 10.1007/s10295-011-1014-8).
20. Prabakaran G., Balaraman K., Hoti S.L., Manonmani A.M. A cost — effective medium for the large-scale production of. *B. sphaericus* H5a5b (VCRC) for mosquito control. *Biological Control*, 2007, 41(3): 379-383 (doi: 10.1016/J.Biocontrol.2007.02.004).
21. Pearson D., Ward O.P. Effect of culture conditions on growth and sporulation of *Bacillus thuringiensis* subsp. *israelensis* and development of media for production of the protein crystal endotoxin. *Biotechnol. Lett.*, 1988, 10: 451-456 (doi: 10.1007/bf01027055).
22. Kalmykova G.V., Cheshkova A.F., Akulova N.I. *Sibirskiy vestnik sel'skokhozyaystvennoy nauki*, 2020, 50(2): 44-51 (doi: 10.26898/0370-8799-2020-2-6) (in Russ.).
23. Smith R.A. Effect of strain and medium variation on mosquito toxin production by *Bacillus thuringiensis* var. *israelensis. Canadian Journal of Microbiology*, 1982, 28(9): 1089 (doi: 10.1139/m82-162).
24. Gladstone G.P., Fildes P.A. A simple culture medium for general use without meat extract or peptone. *British Journal of Experimental Pathology*, 1940, 21(4): 161-173.
25. Lecadet M.-M., Dedonder R. Biogenesis of the crystalline inclusion of *Bacillus thuringiensis* during sporulation. *European Journal of Biochemistry*, 1971, 23(2): 282-294 (doi: 10.1111/j.1432-1033.1971.tb01620.x).
26. Bertani G. Studies on lysogenesis I. The mode of phage liberation by lysogenic *Escherichia coli. Journal of Bacteriology*, 1951, 62(3): 293-300 (doi: 10.1128/jb.62.3.293-300.1951).
27. Tikhonovich I.A., Ermolova V.P., Grishechkina S.D., Romanova T.A. *Shtamm Bacillus thuringiensis var. thuringiensis № 800/15 v kachestve sredstva dlya polucheniya entomotsidnogo preparata. Patent St. Petersburg, GNU VNII sel'skokhozyaystvennoy mikrobiologii RU 2514211 S1. Zayavl. 10.10.2012. Opubl. 27.04.2014. Byul. № 12* [The strain of *Bacillus thuringiensis* var. *thuringiensis* No. 800/15 as a means for obtaining an insecticide preparation. Patent Petersburg, GNU All-Russian Research Institute of Agricultural Microbiology RU 2514211 C1. Appl. 10/10/2012. Publ. 04/27/2014. Bull. № 12] (in Russ.).
28. Tikhonovich I.A., Ermolova V.P., Grishechkina S.D., Romanova T.A. *Shtamm Bacillus thuringiensis var. darmstadiensis № 25 v kachestve sredstva kompleksnogo vozdeystviya na vrednykh zhestkokrylykh nasekomykh i fitopatogennye griby. Patent St. Petersburg, GNU VNII sel'skokhozyaystvennoy mikrobiologii RU 2514211 S 1. Zayavl. 26.12.2012. Opubl. 27.04.2014. Byull. № 12*

[The strain of *Bacillus thuringiensis* var. *darmstadiensis* No. 25 as a means of complex action on harmful beetles and phytopathogenic fungi. Patent Petersburg, GNU All-Russian Research Institute of Agricultural Microbiology RU 2514211 C 1. Appl. 12/26/2012. Publ. 04/27/2014. Bull. № 12] (in Russ.).

29. Grishechkina S.D., Ermolova V.P., Minina G.N., Safronova V.I., Bologova E.V. *Metodika. Kollektsiya shtammov bakteriy-simbiontov vrednykh nasekomykh i gryzunov, prigodnykh dlya biokontrolya chislennosti vrediteley sel'skokhozyaystvennykh rasteniy* [Methodology. Collection of strains of bacteria-symbionts of harmful insects and rodents suitable for biocontrol of pests of agricultural plants]. St. Petersburg, 2014 (in Russ.).
30. Abbott W.S. A method for computing the effectiveness of insecticide. *Journal of Economic Entomology*, 1925, 18(2): 265-267 (doi: 10.1093/jee/18.2.265a).
31. *Metody eksperimental'noy mikologii* /Pod redaktsiey V.I. Bilay [Methods of experimental mycology. V.I. Bilay (ed.)]. Kiev, 1982 (in Russ.).
32. Dospekhov B.V. *Metodika polevogo opyta* [Methods of field trials]. Moscow, 1985 (in Russ.).
33. Nickerson K.W., Bulla Jr. L.A. Physiology of sporeforming bacteria associated with insects: minimal nutritional requirements for growth, sporulation, and parasporal crystal formation of *Bacillus thuringiensis*. *Appl. Microbiol.*, 1974, 28(1): 124-128 (doi: 10.1128/AEM28.1.124-128.1974).
34. Biryukov V.V. *Osnovy promyshlennoy biotekhnologii* [Fundamentals of industrial biotechnology]. Moscow, 2004 (in Russ.).
35. Jouzani G.S., Valijanian E., Sharafi R. *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. *Appl. Microbiol. Biotechnol.*, 2017, 101(7): 2691-2711 (doi: 10.1007/s00253-017-8175-y).
36. Domínguez-Arrizabalaga M., Villanueva M., Escriche B., Ancín-Azpilicueta C., Caballero P. Insecticidal activity of *Bacillus thuringiensis* proteins against coleopteran pests. *Toxins (Basel)*, 2020, 12(7): 430 (doi: 10.3390/toxins12070430).
37. Tsarenko I.Yu., Roy A.A., Kurdish I.K. *Microbiol. zhurn.*, 2011, 73(2): 13-19.
38. Matsumoto T., Sugiura Y., Kondo A., Fukuda H. Efficient production of protopectinases by *Bacillus subtilis* using medium based on soybean flour. *Biochemical Engineering Journal*, 2000, 6(2): 81-86 (doi: 10.1016/s1369-703x(00)00079-6).
39. Devidas P.C., Pandit B.H., Vitthalrao P.S. Evaluation of different culture media for improvement in bioinsecticides production by indigenous *Bacillus thuringiensis* and their application against larvae of *Aedes aegypti* Scientif. *The Scientific World Journal*, 2014, 2014: 273030 (doi: 10.1155/2014/273030).
40. Anderson R.K.I., Jayaraman K. Influence of carbon and nitrogen sources on the growth and sporulation of *Bacillus thuringiensis* var *galleriae* for biopesticide production. *Chem. Biochem. Eng. Q.*, 2003, 17(3): 225-231.
41. Sarrafzadeh M.H. Nutritional requirements of *Bacillus thuringiensis* during different phases of growth, sporulation and germination evaluated by Plackett-Burman method. *Iran. J. Chem. Chem. Eng.*, 2014, 31(4): 131-136 (doi: 10.30492/IJCCE.2012.5936).
42. Saberi F., Marzban R., Ardjmand M., Shariati F.P., Tavakoli O. Optimization of culture media to enhance the ability of local *Bacillus thuringiensis* var. *tenebrionis*. *Journal of the Saudi Society of Agricultural Sciences*, 2020, 19(7): 468-475 (doi: 10.1016/j.jssas.2020.08.004).
43. Pustake S.O., Bhagwat P.K., Dandge P.B. Statistical media optimization for the production of clinical uricase from *Bacillus subtilis* strain SP6. *Heliyon*, 2019, 5(5): e01756 (doi: 10.1016/j.heliyon.2019.e01756).