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ACTIVITY OF INSECTICIDAL *Bacillus thuringiensis* var. *israelensis* STRAINS STORED BY VARIOUS METHODS

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Abstract

Microbiological method of insecticidal pests control is an alternative to chemical pesticides. Insect control agents are based on different microorganisms, which should be stably effective against target pest organism. There are different origins of industrial strains including isolation from natural objects, screening of collections, selection of existing strains, genetic engineering etc. but in all cases beneficial features of the strains should be preserved. In this article, the problems of preserving beneficial features of insecticidal bacteria *Bacillus thuringiensis* var. *israelensis* (BTH_{14}) are discussed. This strain is effective as the pest control agent against larvae of mosquitoes, midges and rice and champignons mosquitoes. Different methods and time of storage of various BTH_{14} strains are shown: 266/2 on meat-peptone agar (MPA) and in corpse of mosquitoes for one year, in sodium chloride crystals for one and a half year; 71 on MPA without reseeding for one year and for two years with reseeding every six months; 87a by cryopreservation for ten years; 87, 404, 19/43 as lyophilized bacteria for 28 years; 7-1/23, 71/82, 19/1 in sodium chloride crystals for 27 years. Culture of BTH_{14} strain was grown on MPA slants at 28-30 °C for 5-7 days until reaching the complete formation of spores and endotoxin crystals. Microscopic analysis was carried out with aniline black dye. Morphological analysis of colonies was performed with colony-purified BTH_{14}. When BtH_{14} was stored by the lyophilization method, the spore culture in a tube on a slant MPA was washed with 5 ml of a 20 % NaCl solution. Then 0.5 ml of the resulting suspension with a titer of 10^7-10^8 CFU/ml was added with a Pasteur pipette into glass ampoules, covered with a sterile swab, then sterile stopper and frozen in a cold bath at a temperature of –22 °C for 1 hour, dried at –45 °C for 23 hours, sealed under vacuum over a gas burner and stored in a refrigerator at 3-5 °C. When using the BtH_{14} storage method in NaCl crystals, 5 ml of 0.9 % saline was added to a tube with spore culture on slant MPA, resuspended, and 0.5 ml of suspension was added to sterile tubes, covered with cotton-gauze stoppers and stored at room temperature. When BtH_{14} was stored by cryopreservation, the spore culture of BtH_{14} was suspended in meat-peptone broth (MBP) with 10 % glycerol. The resulting suspensions (200 μl each) were poured into cryovials and stored at –80 °C. The BtH_{14} titer and larvicidal activity for *Aedes aegypti* mosquitoes were measured once or twice per year. The results showed that the culture of the 266/2 strain after a year of storage in the corpses of mosquitoes *Culex pipiens molestus* or on MPA dissociated with the formation of 0.8 and 1.6 % of the IV S form morphotype which lost activity against *A. aegypti* mosquitoes. The titer of the spores and the larvicidity of the 71 strain were at the initial level after one year of storage on MPA in tubes with paraffinized plugs when reseeded every 6 months. These indicators decreased, respectively, by 12 and 16 % in a year and by 25-27 % after 2 years of storage. Cryopreservation of the 87a strain provided stability of titer and larvicidal activity after 10 years. Thus, the initial titer and larvicidal activity expressed as LC_{50} for *A. aegypti* mosquitoes were 2.74×10^9 CFU/ml and 0.178×10^{-3} %, respectively. After 6 and 10 years, they corresponded to the following indicators: 2.82×10^9 CFU/ml and 0.19×10^{-3} %; 2.72×10^9 CFU/ml and 0.18×10^{-3} %. The BtH_{14} strains 7-1/23, 71/82, and 19/1 were stored in NaCl crystals. After 27 years of storage, their titers and LC_{50} for *A. aegypti* mosquitoes varied within the range of 3.12×10^9-3.52×10^9 CFU/ml and 0.135×10^{-3}-0.150×10^{-3} % as compared to the initial values that were

3.98×10^9-4.29×10^9 CFU/ml and 0.10×10^{-3}-0.11×10^{-3} %, respectively. The 87, 404, and 19/43 strains were stored by the method of freeze drying. After 28 years, their titers and larvicidal LC_{50} for *A. aegypti* mosquitoes remained within 3.32×10^9-3.68×10^9 CFU/ml and 0.11×10^{-3}-0.14×10^{-3} % as compared to the initial values 3.86×10^9-4.45×10^9 CFU/ml and 0.087×10^{-3}-0.103×10^{-3} %, respectively. Thus, the best indicators for preservation of valuable properties of BtH_{14} were obtained when stored in a lyophilized state, in NaCl crystals and using cryopreservation.

Keywords: *Bacillus thuringiensis* var. *israelensis* (BtH14), titer, storage, larvicidal activity

Pest management in vegetable, grains and fruit production present a serious economic problem, with the annual loss of national agricultural production reaches several billion rubles [1]. Biological preparations based on microorganisms of various origins are used to obtain environmentally friendly products, including bacteria, viruses, actinomycetes [1, 2], entomofluoric fungi and entomopathogenic nematodes [3, 4]. However, the review of international practice has indicated a preference for the use of entomopathogenic bacteria *Bacillus thuringiensis* (Bt) [5-7]. They have successfully been used as a safe entomocidal and growth-stimulating agent [8, 9]. In recent years, the number of *B. thuringiensis* varieties reported by domestic and foreign researchers has increased many times over and we now identify over 70 varieties [10]. *Thuringiensis* bacteria form spores, crystalline endotoxin [11-13], thermostable exotoxin [14], and sometimes enzymes with antifungal properties [15]. The advantages of Bt bacteria include their adaptability to streamlined production, wide spectrum of action [16-18], safety for humans and the environment [19, 20] and non-target insects [21-23]. To enable a cost-effective production of environmentally friendly biological products based on *B. thuringiensis* one should provide continuous supply of highly adaptable and virulent strain-producers, which requires the creation of collections and maintaining optimal terms and methods of storage.

It is customary to store bacterial cultures at the stage of their periodical transfer to fresh media. When applying this method, three basic conditions must be met: suitable maintenance medium, ideal storage temperature and transfer rate [24, 25]. Bt crop collections are stored on beveled meat-peptone agar (MPA), fish agar (FA) at a temperature of 3-5 °C in test tubes with unwaxed and waxed cotton-gauze plugs, under mineral oil, in insect corpses, in NaCl crystals, in lyophilized state and by cryopreservation.

Baktokulicid is a highly efficient, environmentally friendly biological product based on BtH_{14} for suppressing mosquitoes and midges (developed at the Russian Research Institute of Agricultural Microbiology in St. Petersburg, which has been tested in various ecological and geographical zones ranging from northern regions to the tropical belt (Russia, Belarus, Ukraine, France, Czechoslovakia, Cuba, India and Sri Lanka). Its activity is not inferior but very often is better than that of foreign analogues [26].

We apply different storage methods to study the integrity of technologically significant properties of BtH_{14} strains used in the production of Baktokulicid. In this paper, we for the first time invoked cryopreservation and used 20% NaCl as a protective medium for storing the lyophilized strains.

The purpose of the study is to assess the viability, productivity and larvicidal ability in a set of strains of *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) when stored by various methods for varying lengths of time.

Techniques. The strain BtH_{14} 266/2 came from the CCEB collection (Culture Collection of Entomopathogenic Bacteria, Prague, Czechoslovakia). Strains 87, 404, 19/43, 7-1 / 23 and 71/82 were isolated from natural substrates (water, sludge and soil), and strains 71, 87a and 19/1 were obtained by the selection method. We studied the activity of BtH_{14} strains by applying different methods and storage periods: 266/2 — on meat-peptone agar (MPA) and in the

mosquitoe corpses *Culex pipiens molestus* 1 year, in NaCl crystals 1.5 years; 71 — on MPA 1 year without passage, 2 years with passages after 6 months; 87a — by cryopreservation for 10 years; 87, 404, 19/43 — in a lyophilized state for 28 years; 7-1/23, 71/82, 19/1 — in NaCl crystals for 27 years.

BtH_{14} culture was grown in solid nutrient MPA or FA media at a temperature of 29±1 °C until spores and crystals formed. When studying the morphological types of colonies, the culture was scattered in FA by using the depleting smear method. The microscopy was conducted by using black aniline dye [26] on day 7. Strain productivity was measured in yeast-polysaccharide media when grown by the in-depth method in Erlenmeyer flasks placed on a shaker with aeration (220 rpm) for 72 hours at 28 °C. The cell titer was measured by the conventional method of serial dilution with FA seeding.

Larvicidal activity was assessed by the WHO method [10, 28] on the *Aedes aegypti* larvae of instar IV. A suspension of the culture fluid (CF) was prepared by diluting in tap water 200, 400, 800, and 1600 thousand times, which corresponded to the conditional content of CF 0.5×10^{-3}; 0.25×10^{-3}; 0.125×10^{-3}; 0.0625×10^{-3} %, or 5.0; 2.5; 1.25; 0.625 μl CF/l. Portion of 50 ml of suspension was poured into Petri dishes at the appropriate dilution rate and 25 mosquito larvae were placed in there. Petri dishes were placed in a thermostat at 28-30 °C for 24 hours, after which the corpses were counted. The mortality rate for each concentration corrected for mortality under control was calculated using the formula:

$$X = (M_o - M_к)/(100 - M_к) \times 100\ \%,$$

where M_o and $M_к$ are the arithmetic mean values of the number of corpses in the test and control, respectively. The obtained data were used to calculate LC_{50}, expressed as the percentage of larvae killed by the Kerber formula [29]:

$$\lg LC_{50} = \lg C_M - \sigma\ (\sum X_2 - 0.5),$$

where C_M is maximum tested concentration of the preparation σ is the logarithm of the ratio for each previous dilution to the next dilution (logarithm of the multiplicity of dilutions) $\sum X_2$ is the sum of the ratio of the number of corpses to the total number exposed for the appropriate dilution.

Strains 87, 404 and 19/43 were lyophilized in 1988 by the following scheme. The culture was grown in the skewed MPA for 7 days, then the biomass was washed off with 5 ml of 20% NaCl, and a bacterial suspension with a titer of 10^7-10^8 CFU/ml was obtained. A portion of 0.5 ml suspension was transferred to glass tubes using a Pasteur pipette, and covered with a sterile cotton wool ball and then with a cotton plug. The interval between transferring the suspension to the tube and lyophilization was reduced to a minimum (max. 1 hour). The culture was frozen in a cold bath at a temperature of –22 °C for 1 h, then dried at –45 °C for 23 h after removing cotton plugs. The tubes were vacuum-sealed over a gas burner and put for storage in the fridge at 3-5 °C.

When BtH_{14} was stored in NaCl crystals, the culture was grown in MPA at a temperature of 30 °C for 5-7 days until the formation of spores and endotoxin. Physiological saline solution (5 ml) was introduced into the tube with the culture, then the biological material was carefully ground in a loop to obtain a homogeneous suspension, and 0.5 ml were added to the biological tubes, which were closed with ordinary cotton-gauze plugs and stored at 18-22 °C. Each sequence was repeated 20 times. We applied cryopreservation to freeze the culture at the stationary growth phase in 10% glycerol and placed it into the plate storage system (Liconic Instruments, Liechtenstein) at 80 °C [28]. To monitor BtH_{14} viability after freezing and assess baseline values of productivity and larvicidal activity, one of the replicates was de-frozen at 37 °C for 3 min and transferred to FA for further manipulations.

The obtained data were processed by the variance analysis method [29]

at 95% confidence interval. In the tables, the mean (*M*) and standard error of the mean (±SEM) are shown.

Results. The following changes in the morphological composition of the population of BtH_{14} 266/2 strain stored by three methods (Table 1) were observed. The colonies of four morphotypes were identified when disseminating to MPA: I-RS forms are grayish-white colonies with a slightly pink shade, rounded or irregularly rounded, flat, with a finely rough surface; spores and crystals of endotoxin were formed after 5 days. II-RS forms (pigmented) are lilac-pink colonies; no pigment was released into the medium. III-R forms are dull white, dry, wrinkled, flat, round colonies; the process of spore and crystal formation in agar media finished after 3 days. IV-S forms are creamy-beige colonies with blade-rugged edges; the chains of vegetative cells (deformed in many cases) were found in the 6-day culture placed in MPA. The ratio of colonies of various morphological types varied depending on the method of storage. I-RS phenotypes typical for the population ranged from 70.4 to 98.6%, whereas morphologically changed phenotypes ranged from 0.1 to 28.8% (see Table 1). The greatest variability was observed for the strain stored in the *C. pipiens molestus* larvae corpses. A total of 28.8% and 0.8% of III-R and IV-S colonies, respectively, were identified in addition to the dominant I-RS morphotype.

1. Natural variability of BtH_{14} 266/2 strain depending on the storage method (laboratory test))

Storage method and period	Viewed colonies	Colonies by morphotype, %			
		I-RS	II-RS (pigmented)	III-R	IV-S
MPA, 1 year	1147	98.3	0.1	–	1.6
NaCl crystals, 1.5 years	654	98.6	–	1.4	–
Culex pipiens molestus larvae corpses, 1 year	974	70.4	–	28.8	0.8

N o t e. MPA — meat-peptone agar. Dashes mean the absence of colonies of the corresponding morphological type. See the Results section for a description of morphotypes.

2. Biological activity of different morphotypes of BtH_{14} 266/2 strain depending on the storage method (*M*±SEM, laboratory test)

Morphotype	Storage method	Spore titre, $\times 10^9$/ml	LC_{50} for L4 *Aedes aegypti*, $\times 10^{-3}$ %
I-RS	MPA	2.55±0.10	0.21±0.04
II-RS (pigmented)		2.43±0.11	0.32±0.04
IV-S		1.23±0.12	0
I-RS	NaCl crystals, 1.5 years	3.23±0.11	0.19±0.04
III-R		2.20±0.12	0.26±0.04
I-RS	*Culex pipiens molestus* larvae corpses	2.28±0.09	0.24±0.04
II-R		1.21±0.12	0.38±0.04
IV-S		0.98±0.10	0

N o t e. See the Results section for a description of morphotypes.

The productivity in a liquid medium and larvicidal ability in relation to *A. aegypti* of different morphological variants was analyzed (Table 2). Colonies of morphotypes I and II in the culture stored in MPA had almost equal productivity: the titers reached $(2.55\pm0.10)\times10^9$ and $(2.43\pm0.11)\times10^9$ CFU/ml of culture liquid, respectively. In larvicidal activity reaching $(0.21\pm0.04)\times10^{-3}$ and $(0.32\pm0.04)\times10^{-3}$ % the morphotype I exceeded the morphotype II by 1.5 times. Morphotype VI culture grew slowly without spore and crystalline endotoxin formation and turned out to be non-pathogenic for *A. aegypti* larvae. The best results were shown by the method of BtH_{14} storage in NaCl crystals. The best results were shown by the method of storage in the corpses of mosquito larvae. Strain 266/2 was not stable and dissociated during storage with the formation of variants with reduced productivity, smooth S-variants lost 100% of their virulence. We managed to isolate by selection a more stable strain BtH_{14}

71, on the basis of which a method was developed for the preparation of a larvicide preparation.

Table 3 includes BtH_{14} strains' titers and activity data obtained by using different storage methods.

3. Biological characterization of 14 BtH_{14} strains after long-term storage (M±SEM, laboratory test)

Strain	Storage method and period	Spore titer, $\times 10^9$/ml		LC_{50} for L4 *Aedes aegypti*, $\times 10^3$ %	
		initial value	after storage	initial value	after storage
71	MPA, 1 year without passages	2.29±0.10	2.83±0.11	0.18±0.02	0.19±0.02
	MPA, 2 years with passsage every 6 months		2.19±0.10		0.21±0.02
87a	Cryopreservation:				
	immediately after freezing	2.74±0.12		0.178±0.02	
	after 3 years		2.78±0.11		0.18±0.02
	after 6 years		2.82±0.10		0.19±0.02
	after 10 years		2.73±0.10		0.19±0.02
87	Lyophilization, 28 years	4.45±0.14	3.68±0.12	0.087±0.02	0.11±0.02
404	Lyophilization, 28 years	4.34±0.12	3.42±0.10	0.092±0.02	0.12±0.02
19/43	Lyophilization, 28 years	3.86±0.11	3.32±0.12	0.103±0.02	0.14±0.02
7-1/23	NaCl crystals, 27 years	4.29±0.11	3.52±0.12	0.10±0.02	0.135±0.02
71/82	NaCl crystals, 27 years	4.18±0.14	3.28±0.13	0.11±0.02	0.142±0.02
19/1	NaCl crystals, 27 years	3.98±0.12	3.12±0.12	0.108±0.02	0.15±0.02

N o t e. MPA — meat-peptone agar..

Comparison of titers and larvicidal activity in different strains of BtH_{14} depending on the method of storage showed (Table 3) that the strain 71 managed to preserve almost all its properties after 1 year storage in MPA with waxed cork. The strains passaged every 6 months lost their properties by 12 and 16% after one year, and by 25 and 27% after two years. The cryopreserved strain 87a managed to preserve its high adaptability to streamlined production and larvicide ability after 10 years of storage. Strains 7-1/23, 71/82 and 19/1 continued to have high titers and larvicidal activity after storage in NaCl crystals for 27 years. The same must be said for the lyophilized strains 87, 404 and 19/43, which were stored in tubes for 28 years. After the lyophilized and cryopreserved BtH_{14} strains have been sown in FA using the depleting smear method for long-term storage, no significant variability was obtained.

The occurrence of atypical forms in the course of storage is quite natural. On the basis of the facts obtained, we conclude that neither cryopreservation, nor lyophilization, nor NaCl crystals should be considered as a basis for contrasting different methods of BtH_{14} storage, on the contrary, the methods should be used complementary to each other, with support of tests for viability and purity, productivity assessment and biotesting prior to the season in which the bacterial larvicidal preparation is to be tried and tested [1].

The stock of Bt strains is constantly replenished. The strains isolated from natural substrates are firstly assessed for entomocidal activity and productivity. The selected active strains are deposited and put into the plate storage system for long-term and loss-free storage of cultures, as well as for accurate labeling, registration and tracking of samples. RCAM database (Russian Collection of Agricultural Microorganisms), which is available on-line (http://62.152.67.70/cryobank/login.jsp), supports about 40 strains of *Bacillus thuringiensis* of various serotypes (depending on the effect on insect pests): var. *thuringiensis* (BtH_1), var. *darmstadiensis* (BtH_{10}), var. *israelensis* (BtH_{14}), var. *kurstaki* (BtH_{3a3b}) [30].

So, as can be seen from the above, the strains of *Bacillus thuringiensis* BtH_{14} can be efficiently stored with the use of cryopreservation, lyophilization and NaCl crystals. Analysis of commercial producers with larvicidal effect for viability, purity, productivity and activity should be performed prior to the sea-

son in which bacterial larvicidal preparations are to be tried and tested at biological factories and laboratories. Earlier studies have shown that the same storage methods could be used for storing *B. thuringiensis* cultures of other serotypes.

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