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RESEARCH ARTICLE

Identifying the Main Technological Parameters for Bio-Product Exemplified by Bacteriophage *Xanthomonas campestris* pv. *campestris* Kl34–UTSAV

Maiorov Pavel[1], Feoktistova Natalya A.[1], Vasilyev Dmitry A.[1], Mallyamova Elvira Sh.[1], Nafeev Alexander A.[1], Toigildin Alexander L.[1]*, Toigildina Irina A.[1], Obukhov Igor L.[2], Shmorgun Boris I.[3]

[1]Ulyanovsk State Agrarian University named after P.A.Stolypin, Ulyanovsk (Russian Federation)
[2]All-Russian Research Institute of Veterinary sanitary, Hygiene and Ecology, Moskau (Russian Federation)
[3]Russian State Center of Quality and Standardization Medicines for Animals and Feed, Moskau (Russian Federation)

Study Area: Ulyanovsk, Russia
Coordinates: 54°19'N; 48°22'E

Key words: Biopreparation, Plant disease

Abstract

Experiments to identify the best purifying method of bacteriophage from the production culture of bacteria was done. The temperature and trichloromethane influence, and also filtration of suspension through a membrane filter with different pore sizes were distinguished. It is established that suspension purifying from bacterial cells by filtration through membrane filters with a pore size of 0.22 micron turn out the best purification method. Under the given time optimum ratio of the result (lytic activity of bacteriophage) and hourage were obtained. The selection of the optimum ratio of phage and bacterial culture for cultivation showed that the best ratio are 1:2 and 1:3. Under the given parameters we obtained similar results. As optimum temperature of cultivation of bacteriophage temperature of 20-32°C was established, at which bacteriophage activity is preserved.

Introduction:

PBacterial diseases of plants are the important reason for the reduction in agricultural yield, as till date, there is no effective means of controlling them. Vascular bacteriosis of crucial, caused by bacteria Xanthomonas campestris pv. campestris, affects almost all known plants, referring to the family crucial (Jensen *et al.*, 2010; Ignatov *et al.*, 2016). Standard means of control of given diseases do not provide satisfactory control of diseases, especially when weather conditions foster spread of agents (Massomo *et al.*, 2004; Francisco *et al.*, 2011). Nowadays bacteriophage appliance for identification and control of agents of bacterial diseases of plants is an invasive stream. Therefore bacteriophages can be used as an effective antibacterial measure (Clokie *et al.*, 2011; Renu *et al.*, 2017). The use of phage and phage identification is one of the most attractive alternatives of existing methods Bacteriophages are the viruses, which specifically infect bacteria, their reduplication leads to lysis of their bacterial host and release of the new- formed phage particles. Phage therapy was not studied for bacteria *Xanthomonas campestris pv. campestris*, however, there are vast data available regarding the use of given methods for other bacteria (Loc-Carrillo & Abedon, 2011; Jones *et al.*, 2012; Balogh *et al.*, 2018).

The appliance of phage biopreparations in different methods (including the reaction of phage titer growth) allows to exert control and analyze the quantitative and qualitative composition of isolated bacteria, it takes less time unlike classical bacteriological methods (Feoktistova, *et al.,* 2018). Phage diagnosis as one of the methods of indication and identification allows to determine the affiliation of studied bacteria not only with specific genus but to the species and even phage var (Chugunova & Tatarnikova, 2016).

However, it is important to note that the selection of bacteriophages forming part of biopreparation for indication and identification of bacteria, requires their deep research and determination of optimum interoperation specifications of phage-bacteria with the aim to minimize the development of their resistance of bacteria to the used bacteriophages (Jones *et al.*, 2012).

The aim of this study was to select an optimal technological parameters for biopreparation production on the basis of earlier isolated bacteriophages.

Materials and methods:

For this piece of work 10 isolates of bacteriophages X.campestrispv. campestris (Kl9–ULSAU, Kl13–ULSAU, Kl20–ULSAU, Kl21–ULSAU, Kl22–ULSAU, Z2–ULSAU, Z4–ULSAU, Z7–ULSAU, Kl33–ULSAU, Kl34–ULSAU, S4–ULSAU), isolated from samples of soil and cabbage with the signs of damage from black rot from the fields of

*Corresponding Author: atoigildin@yandex.ru

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Ulyanovsk and Ulyanovsk region (Department of Microbiology, Virology, Epizootiology and Veterinary & Sanitary expertise of Ulyanovsk, SAU). As production we used strain *X.campestris pv., campestris* Xc2, considering that it had typical properties for such species of bacteria, and also it had the best parameters of growth performances for 24 hours (from 1.6 X 10^8m.k./ml). Additionally to increase reliability in a particular experiment we used bacterial strains B-570 and Xc1.

Mediums and reagents: Bacteriological agar (RMA «Mediums», Makhachkala), dry enzymic peptone (HiMedia), tryptone (HiMedia), yeastrel (HiMedia), medium LB (tryptone - 10 g/l, yeastrel – 5 g/l, NaCl - 10 g/l), sodium chloride (LLC «UlChem»), glucose (HiMedia).

Instruments and equipment: Laaboratory bacteriological ware, heated bath, mercury thermometer, distiller, siccative sterilizing cabinet SSC – 80, digester GK-100-3, minus, and domestic fridges, thermostat-80M-2. The research was carried out on the basis of the given and battle-tested method of members of the above-mentioned departments (Zolotukhin, 2007; Feoktistova *et al.*, 2018).

Results and Discussion :

The main technological parameters of production and control of phage biopreparations are such parameters as qualitative ratio of phage to bacterial crop, optimum ratio of passage time to phage activity, the temperature of cultivation to the purifying method of bacteriophage from production culture of bacteria without change of its main biological properties.

The best purifying method of bacteriophage from production culture of bacteria was determined by studying the stability of bacteria to the temperature (comprised between 56-68°C) and to trichloromethane (in concentration 1:5, 1:10 & 1:20). Besides we carried out a range of experiments with filtration of studied suspension through membrane filters using different pore sizes (0.1 – 0.45 micron). All presented parameters were selected by proper measurements. As a bacterial test-cultures, we used strains of bacteria *X.campestris pv. campestris* B-570, Xc1 and Xc2.

For the study of stability of bacteria *X.campestris pv. campestris* to the temperature test-tube with the suspension of bacterial culture in concentration of 107-108 m.k./ml were heated on a heated bath at the temperature of 56-68°C at 2°C intervals during 10-30 min. at every 10 min. intervals. After bacterial inoculation, on Petri dishes, it was seeded and during 48 hrs we registered growth and absence of growth of studied strains. The results of conducted research are shown in picture 1.

Received data indicate that heating of bacterial cultures during 10 minutes intervals does not give necessary results, as all studied strains showed stability to the temperature (in the range of temperatures 56-68°C) in this time span (table-1). While heating during 20 minutes, strains Xc1, Xc2 showed stability to temperatures down to 62°C (Plate-1), strain B-570 was not stable at the temperatures of 60°C (table-2). At exposition time equal to 30 min. all studied strains showed stability to temperatures 56°C (table-3). Thus we consider optimum temperature for the inactivation of bacteria *X.campestris pv. campestris* is 62°C at exposition time of 20 min. Wyrefused of heating during 30 min., as during reduction of tempera-tures by various degrees time of the experiment is raising by half.

Table-1: Stability of bacteria *X.campestris pv. campestris* to the temperature (oC) (Exposition time 10 minutes)

Temp/Strain	56°C	58°C	60°C	62°C	64°C	66°C	68°C
B-570	+	+	+	+	+	+	+
Xc1	+	+	+	+	+	+	+
Xc2	+	+	+	+	+	+	+

Table-2: Stability of bacteria *X.campestris pv. campestris* to the temperature (oC) (Exposition time 20 minutes)

Temp/Strain	56°C	58°C	60°C	62°C	64°C	66°C	68°C
B-570	+	+	-	-	-	-	-
Xc1	+	+	+	-	-	-	-
Xc2	+	+	+	-	-	-	-

Table-3: Stability of bacteria *X.campestris pv. campestris* to the temperature (oC) (Exposition time 30 minutes)

Temp/Strain	56°C	58°C	60°C	62°C	64°C	66°C	68°C
B-570	+	-	-	-	-	-	-
Xc1	+	-	-	-	-	-	-
Xc2	+	-	-	-	-	-	-

Table-4: Sability of bacteria *X.campestris pv. campestris* to the thrichloromethane depending on concentration & exposure time

Temp	Concentraion 1/5			Concentraion 1/10			Concentraion 1/20			
	10*	20*	30*	10*	20*	30*	10*	20*	30*	
B-570	+	-	-	+	-	-	-	-	-	*minutes
Xc1	+	+	-	+	-	-	-	-	-	
Xc2	+	-	-	+	-	-	+	-	-	

Study of bacteria stability *Xanthomonas campestris pv. campestris* to trichloromethane was carried out in the following way. Into test-tubes with the suspension of bacterial cultures in a concentration of 107-108m.k./mlwe added trichloromethane in the concentration of 1/5, 1/10 or 1/20, after we shook the test-tube actively during 5, 15 and 25 min. Thus exposition time was 10, 20, and 30 min. at the end of time exposures, the supernatant was selected and seeded on Petri dishes. seedings were cultivated at a temperature of 28°C during 48 hr. The results of the conducted experiments are shown in table-4.

Studied bacteria showed stability to the influence of thrichloromethane in concentration 1/5 for 20 minutes, in a concentration of 1/10 during 10 minutes, and in a concentration of 1/20 for 10 minutes. We considered that optimum parameters of time of conducted experiment and

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Table-5: Lytic activity of bacteriophage after purifying of suspension from bacteria by few methods

Bacteriophage	Throchlorometh. (1/10, 20 conc.)	Temperature 62°C, 20 min.	Filtration, Pore size 0.1 micron	Filtration, Pore size 0.22 micron
K19ULSAU	1.3X 10^8±0.1 X 10^8	1.3X 10^7±0.1 X 10^7	1.5X 10^6±0.2X 10^6	1.8X 10^8±0.1 X 10^8
K113UL SAU	1 .2X 10^7±0.1 X 10^7	1 .2X 10^6±0.1 X 10^6	1.0X 10^6+0.1 X 10^6	2.4X 10^7±0.2X 10^7
K120UL SAU	1.1 X 10^7±0.2X 10^7	1.1 X 10^6±0.2X 10^6	1.3X 10^5±0.2X 10^5	1.2X 10^7±0.1 X 10^7
K121ULSAU	1.2X 10^8±0.1 X 10^8	1.0X 10^8±0.2X 10^8	1.3X 10^7±0.1 X 10^7	1.4X 108±0.1 X 10^8
K122ULSAU	1 .3X 10^6±0.1 X 10^6	1 .5X 10^5±0.1 X 10^5	1 .1 X 10^5±0.2X 10^5	1 .8X 10^6+0.3 X 10^6
Z2ULSAU	1.8X 10^5±0.3X 10^5	1.4X 10^5±0.2X 10^5	1.4X 10^5±0. 1 X 10^5	2.5X 10^6±0.2X 10^6
Z4ULSAU	1.6X 10^6±0.2X 10^6	1.2X 10^6±0.3X 10^6	1.1 X 10^6±0.2X 10^6	2.1 X 10^7±0.4X 10^7
Z7ULSAU	1.7X 10^6±0.1 X 10^6	1.5X 10^6±0.2X 10^6	1.2X 10^5±0.2X 10^5	1.5X 10^7±0.2X 10^7
K133ULSAU	1 .1 X 10^7±0.1 X 10^7	1 .2X 10^6±0.2X 10^6	1 .6X 10^5±0.5X 10^5	3 .4X 10^7±0.1 X 10^7
K134ULSAU	1 .5X 10^7±0.2X 10^7	1 .2X 10^7±0.1 X 10^7	1 .8X 10^6±0.4X 10^6	2.2X 10^8±0.1 X 10^8
S4UL SAU	1 .0X 10^7±0.1 X 10^7	1 .4X 10^7±0.1 X 10^7	1 .5X 10^6±0.2X 10^6	1 .5X 10^8+0.3 X 10^8

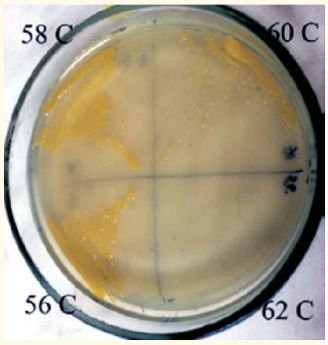

Plate-1:- Bacteria growth *X.campestris pv. campestris* Xc2 after heating during 20 min. (medium LB, 48 hrs 28°C)

quantity of expended reagents are the time of exposition 20 minutes and concentration of thrichloromethane 1/10.

Table-6: Dependence of bacteriophage titer on passage time (in terms of phage K134—ULSAU)

Bacteriophage bacteriophage	Passage time. hours	Lytic activity of By Gratia. BFU/ml
	8	1.2X 10^4±0.2X 10^6
	12	1.3X 10^5±0.2X 10^7
	16	1.5X 10^6±0.4X 10^7
K134-ULSAU	20	1.0X 10^7±0.2X 10^8
	24	2.4X 10^8±0.1X 10^8
	28	3.4X 10^8±0.2X 10^8
	32	1.1X 10^8±0.1X 10^8
	36	3.9X 10^7±0.2X 10^7

Table 7 — Dependence of bacteriophage titer on quantity of production culture of bacteria *X. campestris v. campestris* Xc2 (in terms of phage K134—ULSAU)

Quantity of added indicator culture ml on 0.2 ml phage				
0.2	0.4	0.6	0.8	1.0
1.6x10^7± 0.1x10^7	1.1 X 10^8± 0.2x10^8	2.3x10^8± 0.3x10^8	2.1x10^7± 0.1x10^7	3.3x10^6± 0.2x10^6

Table-8: Optimum temperature parameter of cultivation of bacteriophage during 24 hours (in terms of phage K134—ULSAU)

Phage	12°C	16°C	20°C	24°C	28°C	32°C	36°C
B-570	-	-	+	+	+	-	-

Further experiments with the aim to study influence of earlier described methods of inactivation bacteria on lytic activity bacteriophages and comparison of given methods with a filtrationthrough membrane filters with the pore size of 0.1 micron and 0.22 micron were carried out. It should be pointed out that we didn't use membrane filters with pore size 0.45 micron as after filtration we noted the growth of the bacterial culture.

In test-tubes containing 4.5 ml of culture fluid LB, targetted bacteriophage was seeded (0.2 ml) with master seed strain of bacteria *X.campestris pv. campestris* Xc2 (0.2 ml) and cultivated at the temperature of 28°Cduring 24 hours. After that we carried out handling of suspension by thrichloromethane , temperature, and filtration through membrane filters with pore size 0.1 and 0.22 micron. Further lytic activity of studied bacteriophage was determined by the agar-layer method.

While identifying the best method of purifying suspension from bacterial cells is filtration through membrane filters with a pore size of 0.22 micron. During the use of filters with the pore size of 0.1 micron, a decrease of lytic activity of studied bacteriophages was evidenced (in specific cases from 108 to 106BFU/ml). It is possible that the reason for this phenomenon is quick pore impurity that led to a decrease in the carrying capacity of filters. Treatment by temperature also had affected lytic activity in a negative way almost on all studied bacteriophages (exception is bacteriophage Kl21–ULSAU). More close results to filtration (pore size 0.22 micron) were recorded during treatment of thrichloromethane, however, at specific bacteriophages we observed a noticeable reduction of lytic activity (Z2–ULSAU, Z4–ULSAU, Z7–ULSAU, Kl34–ULSAU, S4–ULSAU). In this view as the most acceptable method of purifying suspension from bacteriophage cells we consider filtration through membrane filters with pore size 0.22 micron. This method will be used in further experiments.

The following stage of our research was on the determination of optimum ratio of passage time and bacteriophage activity, for this aim, we added 0.2 ml of production culture of bacteriophage *X.campestris pv. campestris* Xc2 and 0.2 ml of studied bacteriophage into test tubes with liquid medium. Concurrently, we set control test tubes of the production culture of bacteria and separately of bacteriophage. Cultivation was carried out in thermostat at the temperature of 28°C. Exposition time was selected by experimental way beginning with the middle of exponential phase of growth of bacteria *X.campestris pv. campestris* Xc2 at 4 hours interval (8h±0.3h). After cultivation obtained suspensions, containing bacteriophages, were filtered with the appliance of membrane filters with pore size of 0.22 micron and determined lytic activity by Gratia method (table-6).

It was revealed that the average optimum of the cultivation time of studied bacteriophages at the temperature of 28°C is within 24-28 hrs. In terms of bacteriophages Kl34–ULSAU (table-6) it was seen that within this range of considerable change of lytic activity didn't reveal, due to which as technological parameters during the production of phage preparation, we consider passage time of 24 hrs. It must be noted that during cultivation, 32 hrs of reducing lytic activity of bacteriophages is noticed.

For determination of the qualitative ratio of phage and production culture into separate test-tubes with liquid medium LB in the volume of 4.5 ml we added 0.2 ml of studied bacteriophage (all in 5 test tubes). Further, we added the production culture of bacteria. *X.campestris pv. campestris* Xc2 into a test tube in the concentration of 107-108m.k./ml in volume from 0.2 to 1 ml at 0.2 ml intervals (Parameters were selected experimentally). Concurrently we produced control of bacteriophages and production culture of bacteria. Further test-tubes were cultivated in a thermostat at the temperature of 28°C during 24 hrs. After cultivation, the content of test-tubes was filtered with the appliance of membrane filters with pore size 0.22 micron and determined lytic activity by Gratia method (table-7).

As per the results, it was established that a more optimum ratio of phage and production culture of bacteria for studied bacteriophages are1:2 and 1:3, as evidenced by submitted data in table-7. At a given ratio of phage and bacterial culture almost identical results were obtained. As optimum production parameter, the ratio of phage and bacterial culture 1:2 was chosen, as on receipt of similar results this ratio allowed to save spent recourses.

For the determination of optimum temperature parameters of the cultivation of studied bacteriophages, we added 0.2 ml of studied bacteriophage and 0.2 ml of culture, bacteria *X.campestris pv. campestris* Xc2 in concentration of107-108m.k./ml into test tubes with liquid medium LB in volume of 4,5 ml. Concurrently, we set a control probe of production culture and separately each of bacteriophages. Cultures were cultivated in thermostat during 24 hours at different temperature settings from 12°C to 36°C at 4°C intervals (parameters were selected experimentally). The results of the conducted research are shown in table-8. The Presence of lysis was determined by the absence of turbidity of the medium.

Conducted research showed that the range of optimum temperature of cultivation of studied bacteriophages was within 20–28°C. Bacteriophage Kl34–ULSAU showed positive results in a range of temperatures 20–32°C. It must be noted that after the cultivation of test tubes at 12°C apart from the absence of turbidity in studied test tubes, in control test tubes, this testifies the absence of culture growth at such temperature parameters.

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