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Selection of the most productive nutrient media for the cultivation of predatory nematodes

Modes of mass production of entomopathogenic nematodes isolated from various habitats of Akmola and Pavlodar regions were tested. As a result of the selection of nutrient media and cultivation conditions of entomopathogenic nematodes, the largest populations of nematodes (more than 100,000 young individuals/ml) were obtained on medium C11 containing yeast extract (23 g/l), egg yolk (13.5 g/l), and corn extract (40 g/l). On the 20th day of cultivation, the maximum concentration of viable nematodes was reached (123 856, 127 572, and 122 469 nematodes/ml for isolates AF 29, AF 57, and KP 76, respectively), which was maintained until the end of the experiments. During the process, the initial concentration of nematodes increased by more than 2 orders of magnitude. When working out the modes of mass production of nematodes on a semi-industrial scale, the populations of isolates AF 29, AF 57, and KP 76 in media amounted to 122 754, 131 784, and 126 521 nematodes per ml. The total volume of the resulting preparation was 50 liters. The obtained results are applicable in crop production in the fight against potato pests.

Keywords: entomopathogenic nematodes, *nutrient media, Steinernematids, Galleria mellonella,* biological control.

Introduction

Entomopathogenic nematodes (EPN) are insect parasitic roundworms. They have the ability to infect over a thousand different insect species. The worms' mutualistic association with the bacteria Photorhabdus and Xenorhabdus is one of their most distinguishing characteristics. Both the larval and adult stages of insects are affected by EPN. An infectious juvenile nematode is the nematode's only free-living stage. The nematode's life cycle begins with the entry into the insect's body cavity by natural openings such as the mouth, anus, spiracles, or thin regions of the cuticle. The nematode enters the insect by the hemocele, where it releases symbiotic bacteria that live in the worm's intestines. These bacteria grow rapidly, generating toxins and hydrolytic exoenzymes, resulting in the carrier's temporary death within two to three days. After that, for numerous generations, nematodes continue to develop in the corpse [1]. Even when single nematode individuals enter the insect body, hundreds of thousands of young individuals are already released into the environment after 10–20 days, allowing them to survive for lengthy periods of time without food while waiting for a new insect host [2]. The most studied representatives are nematodes of the families *Steinernematidae* and *Heterorhabditidae* because of their most promising properties for the use [3]. The difference between these two families is that the first generation of *Heterorhabdtidae* is hermaphrodite, and the next generations can have both hermaphrodite and female and male forms. While in the *Steinernematidae*, all generations are have both sexes [4, 5].

At the moment, methods of *in vivo* and *in vitro* solid and liquid media are widely used in the reproduction of EPN in commercial conditions [6, 7].

The *in vivo* method consists in inoculation (infection) of insects with nematodes in Petri dishes or trays equipped with filter paper. After 2–5 days, infected insects are transferred to White traps to collect young nematodes. After the collection is completed, the concentration of nematodes is carried out and, if necessary, their decontamination. The advantage of the *in vivo* method is that it is the simplest in terms of the necessary qualifications and the necessary equipment, but at the same time, this method remains the least cost-effective of the three currently used [8].

When breeding nematodes by *in vitro* method on a solid medium, nematode eggs are placed on a pure culture of nematode symbiont bacteria. As a nutrient medium, the most effective is considered to be a medium consisting of yeast extract, nutrient broth, vegetable oil, and soy flour, or consisting of peptone, yeast extract, eggs, soy flour, and lard. The resulting medium is impregnated with a sponge and autoclaved. Then, bacteria settle first, and after 3 days, nematodes. After 2–5 weeks, the nematodes are collected by placing a

sponge on a sieve and repeatedly rinsing into a collection tank. At the moment, there are already modifications to this method, which allows automating some steps of the process and increasing the volume of production [7, 8].

The *in vitro* liquid method is the most cost-effective of the three, but at the same time, this method has the highest requirements for the qualification of employees, as well as significant capital investments. This method uses a nutrient medium comprising soy powder, yeast extract, rapeseed, corn oil, thistle oil, egg yolks, casein, peptone, liver extract, and cholesterol. Just like in the solid medium method, bacteria settle first, after which nematodes are added. Nematodes are fermented for 2–3 weeks with aeration, after which they can be removed from the medium by centrifugation [8].

The bacteria *Photorhabdus*, which live in the nematodes *Heterorhabditidae*, and *Xenorhabdus*, which live in the nematodes *Steinermatidae*, are gram–negative gamma-proteobacteria of the Enterobacter family. Their relationship with EPN is extremely specific and, as a rule, each type of nematode is strictly associated with a single type of bacteria. At the same time, the bacteria themselves can be associated with several different types of nematodes at once [9, 10].

This study aims to select the medium that can give the highest reproduction number of EPN. Several mediums were prepared and tested on some isolates of EPN for the study goal.

Experimental

Obtaining pure cultures of symbiotic bacteria of EPN isolates. Approximately 200 nematodes were applied to fifth-instar Galleria mellonella larvae and incubated at 25 °C for 10 hours. To isolate bacteria, Galleria mellonella hemolymphs were applied with strokes to NBTA medium (nutrient agar with the addition of 25 mg of bromothymol blue and 40 mg of triphenyltetrazolium chloride per 1 liter). Also, about 500 nematodes were superficially sterilized with 70 % of ethanol for 3 minutes and crushed in sterile distilled water. Then the extract was applied with strokes to the NBTA medium. Colonies of blue color, grown on cups with NBTA, were cultured once in triton soy broth (TSB) at 25 °C for 48 hours. After three times rinsing with sterilized water by centrifugation of the culture medium at 4000 x g for 2 minutes at 4 °C, the cells were resuspended in a sterilized 100 mM phosphate-salt buffer (PBS, pH 7.4) for subsequent tests.

Selection of a medium for mass production on a laboratory scale. Cultivation of APN isolates AF 29, AF57, and KP78 was carried out in plastic trays containing 50 ml of medium, three repetitions of each medium variant. Table 1 represents the compositions of the media.

Table 1

Compositions of the studied media

Medium	Yeast Extract	$MgSO_4 \cdot 7H_2O$	$MnCl_2 \cdot 4H_2O$	KH_2PO_4	Egg yolk	Corn Extract
C1	2,3	0	0	6	27	40
C2	2,3	0	0,5	6	0	40
C3	23	0	0,5	0	0	40
C4	2,3	0,5	0,5	0	0	0
C5	23	0	0	0	27	0
C6	11,5	0,25	0,25	3	13,5	20
C7	23	0,5	0	6	0	0
C8	11,5	0,25	0,25	3	27	20
C9	2,3	0,5	0,5	6	27	0
C10	11,5	0,25	0,25	3	13,5	20
C11	23	0,5	0,5	0	13,5	40
C12	2,3	0	0	0	0	0
C13	23	0,5	0	6	0	40
C14	23	0	0,5	6	27	0
C15	2,3	0,5	0	0	27	40
C16	46	0,5	0,5	0	54	40
C17	46	0,5	0,5	0	27	20
C18	23	0,5	0,5	0	54	40

Symbiotic bacteria of isolates previously grown in a trypton-soy medium were inoculated into the medium by adding 1 ml ($5 \cdot 10^9$ cells) of culture to each tray. The trays were incubated in a thermostat at 25 °C for 2 days. After 2 days, bacterial suspensions were inoculated with nematodes by adding 5000 two-day-old young individuals of isolates AF 29, AF 57, and KP 76 to each tray (final concentration = 100 young individuals/ml). Cultures of nematode bacteria were incubated for 4 weeks before counting nematode populations. To count nematodes, 1 ml of sample was taken from each tray and diluted 1:500 with water in a glass per 1000 ml. The nematode count was performed using a stereomicroscope with a 16-fold magnification. The calculations were repeated twice. The whole experiment was repeated once (thus, two complete tests).

Development of mass production modes on a semi-industrial scale. Symbiotic bacteria of isolates were cultured in a fermenter with a volume of 5 liters in a trypton-soy medium, g/l: casein peptone 17, potassium phosphoric acid 2-substituted 2.5, glucose 2.5, sodium chloride 5, soy peptone 3. To obtain the seed material, mown agar of microorganisms stored on an agarized trypton-soy nutrient medium was used. The production of bacterial seed material was carried out in Erlenmeyer flasks on a liquid trypton-soy medium with a volume of 0.5 liters. The flasks with media were seeded with a suspension of microorganisms in an amount of 10 % of the volume of the medium. The duration of the process of cultivation of microorganisms in flasks is 48 hours at a temperature of 25 °C. The preparation of nutrient media was carried out directly in the fermenter by mixing nutrient solutions and bringing the volume to the required amount with tap water. The pH was adjusted to 6.8–7.0. The medium was sterilized at 121 (± 1) °C for 30 minutes in an autoclave. The seed material obtained in flasks was sown in a fermenter with a volume of 5 liters and cultivated for 48 hours at a temperature of 25 °C. At the end of cultivation, microscopy was performed to control purity, as well as seeding of the material on an agarized trypton-soy nutrient medium to control purity, also to determine the titer by the Koch method.

Cultivation of isolates AF 29, AF 57, and KP 76 was carried out in plastic trays containing 50 ml of medium C11 (Table 1). Sterilization of the medium in the trays was carried out at 121(± 1) °C for 30 minutes in an autoclave. After cooling the trays to room temperature, symbiotic bacteria of isolates previously grown in a trypton-soy medium were inoculated into the medium by adding 1 ml ($5*10^9$ cells) of culture to each tray. The trays were incubated at 25 °C for 2 days. After 2 days, bacterial suspensions were inoculated with superficially sterilized nematodes by adding 5000 two-day-old young individuals of isolates AF 29, AF 57, and KP 76 to each tray (final concentration = 100 young individuals/ml).

Cultures of nematode bacteria were incubated at 25 °C for 4 weeks. During the incubation period, samples were taken from randomly selected trays every four days to control the development of nematodes. To count nematodes, 1 ml of sample was taken from each tray and diluted 1:500 with water in a glass per 1000 ml. After 4 weeks of cultivation, the trays with each isolate were moved to a plastic container and stored at 4 °C.

Results and Discussion

Both *Steinernematids* and *Heterorabditids* form a mutualistic symbiosis with gram-negative bacteria from the genera *Xenorhabdus* and *Photorhabdus*, respectively, which can occur in two forms, and only one of them — phase I — is important for the effective destruction of host insects. They also support nematode reproduction well compared to phase II bacteria. The bacteria are carried in the intestine at stages IJ, a specialized free-living form of nematodes. IJ look for insect larvae in the soil, invade them and release their symbiotic bacteria in the hemolymph, where they multiply and produce a wide range of toxins and hydrolytic exoenzymes that are responsible for the death and bioconversion of insect larvae into a nutrient substrate that is ideal for the growth and reproduction of nematodes. Nematodes multiply until the supply of nutrients become limited, after which they develop into IJ, which are re-colonized by symbiotic bacteria [11].

To isolate symbiotic bacteria from isolates AF 29, AF57, and KP76, superficially sterilized nematodes and hemolymph of *G. mellonella* infected with nematodes were used as bacterial sources. Both the nematode source and the host hemolymph produced colonies of blue and red bacteria on NBTA medium (Fig. 1 and 2). As a result of the adsorption of bromothymol blue by phase I colonies, lightened zones are formed around them, and the colonies themselves acquire a characteristic color.

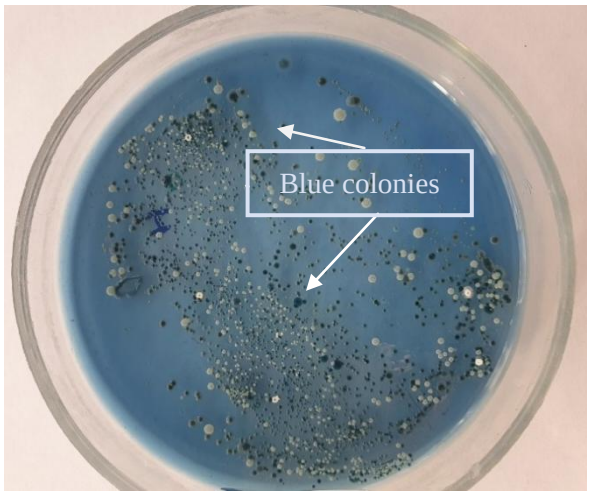

Figure 1. Bacterial isolates grown on NBTA medium from *Galleria mellonella* hemolymph infected with nematode isolate AF 29

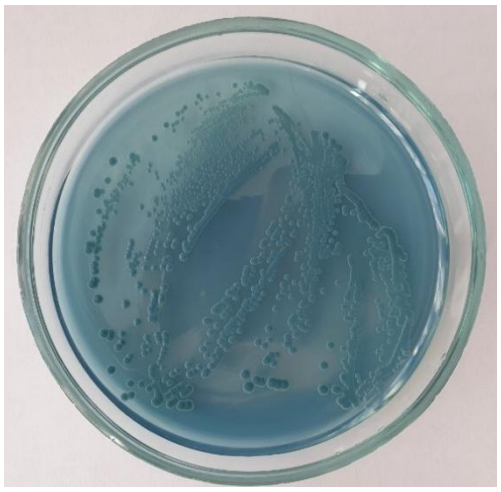

Figure 2. Pure AF 29/1 isolate culture grown on NBTA medium

Tests for catalase and oxidase were carried out to check the genera of bacteria. The results showed that these bacterial isolates belong to the genus *Xenorhabdus*. Table 2 illustrates the biochemical identification of bacteria.

Table 2

Biochemical identification of bacteria from isolates AF 29, AF 57, and KP 76

Parameter	Isolate AF 29/1	Isolate AF 57/1	Isolate KP 76/1	*Xenorhabdus*	*Photorhabdus*	*Serratia*
Gram	–	–	–	–	–	–
Catalase	–	–	–	–	–/+	–
Oxidase production	–	–	–	–	–	–/+
Insecticidal activity	+	+	+	+	+	–
NBTA Colony Color	Blue	Blue	Blue	Blue	Red	Pink-red

The technology of submerged monoxene cultivation *in vitro* is the best option for mass production of entomopathogenic nematodes according to various authors [12, 13], but, at the same time, the best quality of IJ is achieved through *in vivo* cultivation technology. Although some companies produce stages IJ using deep cultivation technology [14], the results obtained are too different, and efforts should be made to improve the available technologies. In particular, the research concerns the development of the environment — the optimization of the environment — necessary for more reliable bioprocess production of IJ. As for the composition of the medium, most sources used complex formulations rich in nutrients, which, among other ingredients, would contain dried egg yolk, lactalbumin, and various vegetable oils and extracts [15, 16].

To optimize the production of EPN, the influence of the composition of the nutrient medium on the populations of nematodes AF 29, AF 57, and KP 76 was studied. Lipids, yeast extract, egg yolk, soy flour, salts, and proteins were used in the study. 18 environments with different compositions were tested. The nutrient media also contained 0.2 % of agar.

As a result of the tests, the largest populations of nematodes (more than 100,000 young individuals/ml) were obtained on medium C11 containing yeast extract (23 g/l), egg yolk (13.5 g/l), and corn extract (40 g/l) (Tab. 3).

During the first 3 days of cultivation, inoculated nematodes did not show any changes. On the 4th day, the restoration of the IJ stages led to a change of IJ4 individuals. For 8 days, adults of the first generation were observed, as well as a large number of eggs and J2 individuals. The most intensive growth of the nematode population occurred between 8 and 12 days of cultivation. On the 20th day, the maximum concentration of viable nematodes (123856 nematodes/ml) was reached, which was maintained until the end of the experiments. Thus, the initial concentration of nematodes increased by more than 2 orders of magnitude during the process.

Table 3

Selection of media for isolates AF 29, AF 57, and KP 76

Medium	Yeast Extract	$MgSO_4 \cdot 7H_2O$	$MnCl_2 \cdot 4H_2O$	KH_2PO_4	Egg yolk	Corn Extract	Nematodes/ml		
							AF 29	AF 57	KP 76
C1	2,3	0	0	6	27	40	89 645	92 334	88 641
C2	2,3	0	0,5	6	0	40	64 354	66 285	63 633
C3	23	0	0,5	0	0	40	84 962	87 511	84 010
C4	2,3	0,5	0,5	0	0	0	57 843	59 578	57 195
C5	23	0	0	0	27	0	46 213	47 599	45 695
C6	11,5	0,25	0,25	3	13,5	20	96 485	99 380	95 404
C7	23	0,5	0	6	0	0	43 617	44 926	43 128
C8	11,5	0,25	0,25	3	27	20	92 764	95 547	91 725
C9	2,3	0,5	0,5	6	27	0	78 273	80 621	77 396
C10	11,5	0,25	0,25	3	13,5	20	86 417	89 010	85 449
C11	23	0,5	0,5	0	13,5	40	123 856	127 572	122 469
C12	2,3	0	0	0	0	0	23 714	24 425	23 448
C13	23	0,5	0	6	0	40	72 142	74 306	71 334
C14	23	0	0,5	6	27	0	49 874	51 370	49 315
C15	2,3	0,5	0	0	27	40	83 475	85 979	82 540
C16	46	0,5	0,5	0	54	40	114 125	117 549	112 847
C17	46	0,5	0,5	0	27	20	116 426	119 919	115 122
C18	23	0,5	0,5	0	54	40	126 412	130 204	124 996

The potential for the effective production of EPN using *in vitro* cultivation technology was recognized since 1930, when Rudolf Glazer developed the first method of artificial cultivation. Studies on the production of EPN in liquid cultures focused on increasing the population, the quality of nematodes and production costs by optimizing the environment and cultivation conditions [16].

Phase I symbiotic bacteria support nematode reproduction well. To work out the modes of mass cultivation of nematodes, fermentations of isolates AF 29/1, AF 57/1, and KP 76/1 were carried out on a 7L fermenter. During fermentation, the growth of symbiotic bacteria in a liquid medium was accompanied by turbidity of the medium, the formation of a grayish-white film and sediment. As a result, culture fluids of symbiotic bacteria with a titer of at least 5×10^9 CFU/ml were obtained, which were used for seeding trays with C11 medium for incubation of nematodes.

As a result of cultivation of nematodes in patches for 28 days, the populations of isolates AF 29, AF 57, and KP 76 in media amounted to 122 754, 131 784, and 126 521 nematodes per ml. After 4 weeks of cultivation, the trays with each isolate were moved to a plastic container and stored at 4 °C. The total volume of the resulting bioproduct was 50 liters.

Conclusions

During the implementation of the project and the reporting period of the obtained experimental data, the following results were obtained with the corresponding conclusions:

As a result of the selection of nutrient media and cultivation conditions of entomopathogenic nematodes, the largest populations of nematodes (more than 100,000 young individuals/ml) were obtained on medium C11 containing yeast extract (23 g/l), egg yolk (13.5 g/l), and corn extract (40 g/l). On the 20th day of cultivation, the maximum concentration of viable nematodes was reached (123 856, 127 572, and 122 469 nematodes/ml for isolates AF 29, AF 57, and KP 76, respectively), which was maintained until the end of the experiments. During the process, the initial concentration of nematodes increased by more than 2 orders of magnitude.

When testing the modes of mass production of nematodes on a semi-industrial scale during 28 days of cultivation, the populations of isolates AF 29, AF 57, and KP 76 in media amounted to 122,754, 131,784, and 126,521 nematodes per ml. After 4 weeks of cultivation, the trays with each isolate were moved to a plastic container and stored at 4 °C. The total volume of the resulting drug was 50 liters.

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Жыртқыш нематодтарды өсіру үшін ең тиімді қоректік ортаны таңдау

Ақмола және Павлодар облыстарының әртүрлі мекендеу ортасынан бөлінген энтомопатогенді нематодтарды жаппай өндіру тәртібі әзірленді. Энтомопатогендік нематодтардың қоректік ортасы мен өсіру жағдайларын таңдау нәтижесінде құрамында ашытқы сығындысы (23 г/л), жұмыртқаның сарысы (13,5 г/л) және жүгері сығындысы (40 г/л) бар с11 ортасында нематодтардың ең көп популяциясы (100 000-нан астам жас/мл) алынды. Өсірудің 20-шы күні өміршең нематодтар ең жоғарғы концентрациясына жетті (123856, 127572 және 122469 нематодтар/мл AF 29, AF 57 және KP 76 изоляттары үшін сәйкесінше), бұл тәжірибелердің соңына дейін сақталды. Процесс барысында нематодтардың бастапқы концентрациясы 2 реттен астамға өсті. Жартылай өнеркәсіптік масштабта нематодтарды жаппай өндіру режимдерін әзірлеу кезінде AF 29, AF57 және KP 76 изоляттарының популяциясы ортада 122 754, 131 784 және 126 521 нематодтарды құрады. Алынған препараттың жалпы көлемі 50 литр болды. Алынған нәтижелер өсімдік шаруашылығында картоп зиянкестерімен күресуде қолданылады.

Кілт сөздер: энтомопатогендік нематодтар, қоректік орта, *Steinernematids*, *Galleria mellonella*, биологиялық бақылау.

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Подбор наиболее продукивных питательных сред для выращивания хищных нематод

Отработаны режимы массового производства энтомопатогенных нематод, выделенных из различных сред обитания Акмолинской и Павлодарской областей. В результате подбора питательных сред и условий культивирования энтомопатогенных нематод наибольшие популяции нематод (более 100 000 молодых особей/мл) были получены на среде С11, содержащей дрожжевой экстракт (23 г/л), яичный желток (13,5 г/л) и кукурузный экстракт (40 г/л). На 20-й день культивирования была достигнута максимальная концентрация жизнеспособных нематод (123 856, 127 572 и 122 469 нематод/мл для изолятов AF 29, AF 57 и KP 76 соответственно), которая поддерживалась до конца экспериментов. В ходе процесса исходная концентрация нематод увеличивалась более чем на 2 порядка. При отработке режимов массового производства нематод в полупромышленных масштабах популяции изолятов AF 29, AF 57 и KP 76 в средах составили 122 754, 131 784 и 126 521 нематод на мл. Общий объем полученного препарата составил 50 л. Полученные результаты применимы в растениеводстве при борьбе с вредителями картофеля.

Ключевые слова: энтомопатогенные нематоды, питательные среды, *Steinernematids*, *Galleria mellonella*, биологический контроль.