Biocatalysis and Agricultural Biotechnology 19 (2019) 101098

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Prospects of using entomopathogenic fungus in development of a biopesticide product with nematicidal activity

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ARTICLE INFO

Keywords:
Biological pest control
Nematodes
Beauveria bassiana strain
Enzymes
Whey
Brewery spent grain and spent grain liquor

ABSTRACT

Entomopathogenic and nematophagous fungi are the recourse for biological control of phytoparasitic insects or various stages of nematodes. The objective of this work was to evaluate the prospects of using an entomopathogenic fungus strain of *Beauveria bassiana* to develop a biological product, in native form and immobilized on a carrier, which would possess, among other characteristics, nematicide activity. In culture suspension based on a mixture of milk whey and brewing spent grain liquor, the product showed catalase and cellulolytic activity, as well as high yield of spore-bearing biomass. Insecticidal activity of the fungus has been determined relative to a test-organism - *Drosophila melanogaster*. Virulence was at 50% with the infective dose of 10^2 CFU. A method of fungus passaging through organism of an insect is proposed as a way of maintaining biological activity. We have found nematocidal activity of the strain of *B.bassiana* with regard to a test organism – bacterial-feeding nematodes of the Rhabditidae family. A correlation dependency has been revealed between biological activity in terms of motile stages of nematodes and the concentration of culture suspension containing enzymes, secondary metabolites and native (own) toxins of entomopathogenic fungus. Use of native, undiluted suspension based on the mixture of milk whey and brewing spent grain liquor ensured 100% death of the number of nematodes within 2 day of incubation. The suggested product is a biodegradable carrier-immobilized form of biopesticide developed using spent grain that ensured viability of infectious units of the strain after storage.

1. Introduction

World agriculture suffers essential yield losses caused by plant pests (Maxmen, 2013). Synthetic pesticides against different phytophages and plant pathogens are ecologically unsafe (Bhattacharjee and Dey, 2014). A promising alternative are bio-pesticide preparations (Lomer et al., 2001). Microorganisms in preparations inhibit phytophages and limit their reproduction (Eilenberg et al., 2001).

Phytohelminths or phytoparasitic nematodes, are highly pathogenic organisms that can often cause a broad infestation of agricultural and forestry crops (Williamson and Gleason, 2003; Perry and Moens, 2006). These open-ground or greenhouse phytophages mostly belong to the Chromodorea class, the Rhabdihida order (Campbell and Kaya, 1999; Zinovyeva et al., 2012). The problems due to soil infestation with phytohelminths are pertinent not only for Russia, but also for other countries (Chitwood, 2003).

Representatives of every fungal or fungus-like order (*Chytridiomycota, Zygomycota, Ascomycota, Basidiomycota, Oomycota*) are known to be capable of attacking motile or nonmotile stages of nematodes one way or another (Whipps and Lumsden, 2001; Lopez-Llorca et al., 2008). The nematicidal activity means, first of all, immobilization of nematodes when exposed to toxins with further introduction of hyphae and degradation (digestion) of organism with help of enzymes (Comans-Pérez et al., 2014). This strategy is primarily typical for nematophagous fungi. Nematoparasitic fungi synthesize a complex of enzymes capable of killing eggs. Nematophagous (carnivorous) fungi synthesize numerous compounds (attractants, toxins, enzymes: proteases, lipases, chitinases), as well as trapping mechanisms against motile stages of nematodes (larvae and sexually mature adult specimens) (Teplyakova and Ananko, 2009; Liu et al., 2009). Well-known biological preparations are based on nematoparasitic and nematophagous fungi as *Paecilomyces* and *Arthrobotrys* (patents: US 5,989,543; CN 101422168; CN 101081982; DE 102005024783; CAN 2059642; RU 2636550; RU 2634390). These biopreparations have a selective capacity of affecting motile and nonmotile stages of certain species of phytohelminths.

Nematodes are known to affect and feed on a large number of plants (Dropkin, 1989). Moreover, all known plants can host one or more species of parasites. The golden nematode *Globodera rostochiensis,* for instance, is a dangerous pest for nightshades. This soil parasite is the

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https://doi.org/10.1016/j.bcab.2019.101098
Received 6 November 2018; Received in revised form 14 February 2019; Accepted 16 March 2019
Available online 21 March 2019
1878-8181/

second most harmful potato pest after Colorado beetle. Therefore, important aspects include strain selection and finding environmentally safe approaches to developing a biological pesticide that will, above all, demonstrate efficacy in fighting various species of pests and be biodegradable.

Phytoparasitic nematodes are microscopic soil phytophages that develop in the rhizosphere, on roots and tubers. Biological pest control requires the product to be degradable and convenient in use for soil treatment. Such convenience in use can be ensured by immobilization of the product using an appropriate agricultural carrier. A lignin-carbohydrate carrier, being part such a pesticide, can also perform a fertilizing function (Patent US 2009227452). Carrier-immobilized biopreparations ensure activity and viability of a microbe culture during storage and usage in agrobiocenosis by being resistant to stresses, including those caused by eco-toxicants (Sharapova and Garabadzhiu, 2015).

Entomopathogenic fungi, along with nematophagous fungi, are of interest due to pertinency of biological control of phytophages (Whipps and Lumsden, 2001; Charnley and Collins, 2007). Biopreparations based on entomopathogenic fungi potentially could be used for biological control of insects that damage above-ground portion of plants. Entomopathogens affect insects by synthesizing cuticle-degrading enzymes (proteases, lipases, chitinases), as well as toxins and metabolites responsible for contact insecticidal activity (Mitina et al., 1997; Roberts and Leger, 2004; Butt et al., 2009). Pathogenicity of nematophagous and entomogenous fungi is characterized by the factors of virulence and host infection strategy. Virulence factors include toxigenicity and extracellular secretion of hydrolytic enzymes (Wang et al., 2000; Cox et al., 2001; Lopez-Llorca et al., 2008). Representatives of both fungal groups have common pathogenic characteristics, being able to invade hosts through protective barriers and to multiply inside the host organism.

The most researched microbiological pest control agents are strains of *Beauveria bassiana* (Bals.-Criv.) Vuill., entomopathogenic fungi (Kryukov et al., 2007; Wang and Leger, 2013). The insecticide activity of these fungi affects representatives of Lepidoptera, Coleoptera, Hymenoptera, Orthoptera, and Acarina orders at different development stages (larvae and imago) (Ogarkov and Ogarkova, 2000; Charnley and Collins, 2007). Like the majority of entomopathogenic muscardine fungi, *Beauveria bassiana* initiates invasion by germination of a spore (conidium) attached to the cuticle of host insect, as well as under action of the enzyme complex responsible for its infectivity. Inside the insect, fungus multiplies due to nutrient-rich hemolymph, produces hyphal bodies or blastospores that lead to mycosis and death of the host (Xiao et al., 2012). Pathogenicity of these fungi is also characterized by their ability to produce secondary metabolites that accelerate the death of an insect: beauvericin, beauverolids, bassionolides, etc. (Tutelyan and Kravchenko, 1985; Xiao et al., 2012). Prevalence of fungi in different types of soil indicates that rhizosphere may be their habitat (Hu and Leger, 2002). Insecticidal activity of *Beauveria bassiana* strains is well-known and their use against different insects is a proven practice (Donadio et al., 2002; Faria and Wraight, 2007; Federici et al., 2008; Patent US 4925663). However, there are no data on nematocidal activity of these fungi.

The objective of this work is to assess the prospects of using the *Beauveria bassiana* entomopathogenic fungus strain to develop a biopreparation in native and carrier-immobilized form that possesses, among other characteristics, nematocidal activity.

2. Materials and methods

2.1. Cultivation of Beauveria bassiana strain

The work was carried out with the *Beauveria bassiana* strain (RNCIM[1] F-145). The strain was stored on the Czapek and Saburo's media on agar groups at temperatures between 2 and 4 °C. To get conidia, the strain was stab-inoculated in the Czapek and Saburo's agar medium in Petri dishes (9 cm in diameter) and cultivated for 3–10 days at 24 ± 1 ^{0}C. The strain forms convex round colonies with a well-developed white-colored aerial mycelium.

For submerged cultivation we used dairy and brewery production by-products - milk (curd) whey and spent grain liquor. The strain was cultivated on liquid nutritive media, namely semi-synthetic Czapek medium and mixture (1:1) of whey and spent grain liquor with addition of Tween-80 inducer (0.5%). Composition of the Czapek medium (g/dm^3) was $NaNO_3$ – 3; K_2HPO_4 – 1; $MgSO_4$ – 0,5; KCl – 0,5; $FeSO_4$ – 0,01; saccharose – 20. Composition of brewing spent grain liquor (mg/dm^3) was suspended substances of plant origin (500–1000), fats (5-10), mineral salts (100–300), sugars concentration (traces). Composition of milk whey (%) was dry substances, including residual milk protein (4.2–7.4), fats (0.05–0.4), lactose (3.2–7.8), mineral salts (0.5–0.8). The initial pH value of growth media was 6.3 ± 2. To prepare the inoculum, fungi were grown in Czapek's agar in Petri dishes for 7–12 days at 24 ± 1 ^{0}C. Agar blocks (8 mm in diameter) were cut off the colony growth zone and then were transmitted into 250-ml conical flasks, with culture medium volume of 100 ml. Cultivation was conducted at T = 25 °C in a shaking apparatus (180 shakes/min) for 5–8 days.

Number of infection units was identified based on the number of colony-forming units (CFU) – germinated spores (fragments of mycelium). The content of biomass in the culture suspension was determined by oven dry weight in 5-mL samples (g/dm^3).

In the course of growth, activity of the studied enzymes and secondary metabolites in filtered and native culture suspension was accessed. Catalase activity in strain suspension was determined based on hydrogen peroxide (10%) reaction.

The cellulose-degrading ability of the *B.bassiana* strain was determined by growing the studied culture on mineral agar medium with added 0.1% carboxymethyl cellulose (CMC), introducing it by 0.1 ml. After 3–5 incubation days, at 30 ^{0}C, dishes were filled with 0.1% Congo red solution for 15 min; the dye was then removed, and 1M NaCl solution was filled for 10 min. Dishes were examined for fading, and diameter of fading zones and ratio of fading zone diameter to colony diameter were measured (Teather and Wood, 1982; Solovyeva et al., 2005).

While the strain was being grown on different media, total cellulase activity was determined based on the quantity of reducing sugars (unit/cm^3 or unit/ml) in the culture suspension on the first and fifth day. The method is based on detection of reducing sugars that form when the substrate (chromatographic paper) is exposed to enzymes of cellulolytic complexes (Polygalina et al., 2003). The unit of cellulase activity is such amount of enzyme that forms 1 mg of reducing sugars (in glucose equivalent) while acting upon 50 mg paper within 1 h.

2.2. Determination of insecticidal activity

Drosophila melanogaster flies were used to determine insecticidal activity, as well as for passaging of hyphomycetes through insect's organism. 30 specimens of flies in a batch and in three replications were kept in flasks with nutrient broth. To infect insects, we used pre-dried conidial mass of the *Beauveria bassiana* strain grown on Czapek medium. Flies were infected with preparations equal by weight with predefined concentration of infection units. A 3 day-old culture with minimal sporulation was used for weak infection, and a 10 day-old culture with active sporulation was used for strong infection. This resulted in at least 10 and 100 colony-forming units per individual (CFU), correspondingly. For this, flies put to sleep with carbon dioxide were placed into Petri dishes with fungal biomass for 1 min and were given a

[1] VKPM – Russian National Collection of Industrial Microorganisms (RNCIM).

shake. Then, they were transferred into flasks and kept at temperature of 20 ± 2 ^{0}C. Flies were maintained in 12 h illumination and relative humidity of 60% (Mitina and Sokornova, 2013; Moskalev et al., 2015). The biological activity of preparation was assessed by mortality rate of insects according to Abbott formula (1925).

After infestation, 3 specimens of infested flies were taken from every replication group and were placed into Eppendorf tubes. To determine the infective dose based on CFU/ml, each insect was treated with sterile water with addition of Tween-80 (0.025%), and were inoculated from washings using Koch's Pour Plate Method. To ensure passage through insect organism, infected flies were put into sterile distilled water and held in Eppendorf tubes at T = 20 ± 2 and T = 2-4^{0}C. Development of mycoses was observed on Axiovert 200-M (Carl Zeiss) microscope with 40X magnification.

2.3. Determination of nematocidal activity

The nematocidal activity was assessed by the ability to kill motile stages of free-living soil bacterial-feeding nematodes *Rhabditis* sp. of the Rhabditidae family. The test-organism was pre-grown on the *Escherichia coli* bacteria lawn (RNCIM B-8208). The *Beauveria bassiana* strain was cultivated in submerged culture on fluid media; the biomass was separated from culture native fluid by filtering through the Red Band paper filter. The culture fungal suspension was tested undiluted and diluted with sterile water in ratio of 1:100. Suspension samples of equal volume were distributed between plate wells, where subsequently equal number of nematodes (20–40) were placed in three replications (n = 3) and were kept at T = 20 ± 2^{0}C without lighting. The stereoscopic Olimpus SZ51 microscope was used to monitor inhibition of motility and to record the number of viable nematodes for 2 days after treatment (18 h, 42 h) in %. Sterile water was used for monitoring. Nonmotile nematodes from test media were transferred into water to detect nematostatic effect (once per day) in order to determine efficiency of toxins contained in culture suspension. Live specimens of nematodes can restore motility function after minor toxic exposure to the media.

2.4. Determination of viability of immobilized biopreparation

To get the immobilized form of biopreparation, brewery by-product, spent grain (0.3–1.4 cm fractions), was used as lignin-cellulose-containing carrier of fungal biomass. The main components of spent grain (%) are: protein and fiber - 18–28; ash – 3–6; lignin – 10–12; extractive substances – 35–38. Spent grain was pre-dried at T = 105^{0}C, or was first milled (to 0.2–0.5 cm fraction) and then dried at T = 45 ± 5^{0}C. Pre-treated samples of spent grain were mixed with equal quantities of culture suspension produced on medium with milk whey and spent grain liquor. The resulting mixture of grain and biomass was dried at T = 45 ± 5^{0}C until oven-dry and stored for 40 days at T = 2–4^{0}C. Viability of carrier-immobilized biomass was assessed based on rate of its recovery after wetting of samples with equal amounts of water. Wetted samples were kept for 5 days at T = 20 ± 2^{0}C. Prior to and after wetting, washings from samples with sterile water were made with addition of Tween-80 (0.025%). Inoculation to agar media was carried out by limiting dilution method while registering the number of CFU in 1 g of spent grain carrier.

Obtained empirical data were statistically processed according to conventional for biology methods using Microsoft Excel and Minitab application software suits. Tables and graphs show average values from all experiments with their standard errors with 0.95 probability level. Differences between treated and control samples, were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Cultivation of the Beauveria bassiana strain

The efficacy of biopreparation is caused by its biological activity against pests (phytophages) and productivity of microbial culture in appropriate application conditions. Entomopathogenic fungi are adaptable due to their ability to change qualitative and quantitative composition of enzymes they synthesize depending on the environmental conditions. However, adverse conditions and abrupt change of substrate may cause delays in development of the microorganism and even its death.

Therefore, in order to obtain an efficient biopreparation, it is necessary to use substrates and conditions that affect growth, viability and activity of a biological agent – spore-containing biomass of entomopathogenic fungus. That is why productivity of *Beauveria bassiana* strain was studied when cultivated at different nutrient media (Table 1).

Spores should prevail in the mycelial mass, as spore germination ensures higher rate of the pathological process compared to mycelium growth. Although fungi grow better on carbohydrate-rich media, their long-term cultivation on such media may reduce sporulation. This is demonstrated by empirical data on productivity in case of submerged and submerged-surface cultivation of fungus on two different media.

Number of infection units, biomass yield, and fermentative activity were different on different media. Duration of lag-phase was almost the same for all substrates. All nutrition media demonstrated the catalase and cellulolytic activity of a hyphomycete. However, on Czapek's medium the total cellulolytic activity was 0.88 ± 0.05 and 0.92 ± 0.05 units/cm^3 on 1 and 5 respectively, whereby on milk whey + spent grain liquor it was 1.64 ± 0.05 and 3.21 ± 0.07 units/cm^3 respectively. Complex media contained lactose and whey milk protein, as well as an inducer and residual components of plant origin from spent grain liquor that stimulated not only biosynthesis of enzymes, but also accumulation of biomass. We have found that enzymatic activity increased depending on the biomass indicator ($p \leq 0.05$; $r = 0.81$). Moreover, culture suspension of whey contained significantly more infection units than the semi-synthetic Czapek's medium. Indicators varied by several orders. In case of submerged-surface cultivation, titre of infection units of fungus increased, whereas

Table 1
Cultivation of the *Beauveria bassiana* strain on different media.

Nutrition media	Cellulolytic activity	Catalase activity	Productivity			
			Submerged cultivation (5 days)		Submerged and submerged-surface cultivation (8 days)	
			CFU Titre	Biomass	CFU Titre	Biomass
			n x 10^6 CFU/ml	g/dm^3	n x 10^6 CFU/ml	g/dm^3
Czapek's medium	+	+	2,03 ± 0,15	9,71 ± 0,43	11,58 ± 3,04	12,26 ± 0,32
Milk whey + brewery spent grain liquor + Tween-80	+ + +	+ +	47,32 ± 5,68	13,14 ± 0,58	1804,91 ± 64,04	14,53 ± 0,67

Note: «+» – presence of fermentative activity; «-» – absence of fermentative activity.

biomass indicators showed minor increase. Suspension of mixed whey and spent grain liquor demonstrated higher biomass indices when grown by submerged and surface-submerged culture methods. Thus, cultivation on optimized nutrition medium significantly increases the productivity of hyphomycete due to growth of mycelium mass and blastospores. Composition of nutrition medium affects the synthesis of exoenzymes apart from those evolutionally capable of synthesizing entomopathogenic fungi.

3.2. The insecticidal activity of the Beauveria bassiana strain

To study the insecticidal activity of the *Beauveria bassiana* strain, *Drosophila melanogaster* flies were used which are not phytophages but can be used as a test-organism (Moskalev et al., 2015). Development of the pathological process is influenced by titre of infection units. Two concentrations of conidial mass of fungus were used to treat insects. It was found that when flies are infected, *B. bassiana* strain demonstrated about 50% virulence (Fig. 1). Washings from infected flies revealed that $(1.2 \pm 0.3)x10^2$ CFU/ml of infection units of strain of entomopathogenic fungus is sufficient for treatment of this type of insects in order to ensure 50% mortality of imago (LC50).

Adhesion of dry conidial mass of on the surface of insect's body is less efficient than that of suspension drops. Germ tubes of a fungus may develop on the surface of cuticle or grow into insect's body. Temperature and humidity are the limiting factors for pathological process development. Observation of mycosis development in organism of live specimens of flies separated from replications showed that the latent period, when the parasite should invade host organism and multiply in hemolymph, lasted for 14–20 days.

Active growth of mycelium on body parts of infected or dead insect due to it being submerged in water occurred on the 10th −12th day at $T = 20 \pm 2^0C$ (Figs. 2–5). Insects submerged in water medium at T = 2-4^0C were affected by mycosis significantly later – on 48th - 56th day.

As frequent transfers promote saprophytization of parasitic types of fungi, use of insects as a substrate allows to maintain biological activity of strains in pure culture conditions (isolate of culture). *Drosophila melanogaster* flies infected with mycosis were used to store and passage the fungus. When stab-inoculated onto agar media, hyphomycete from the agar Czapek medium and mycosis-infected flies demonstrated different radial growth rates. Growth rate and colony diameter values were significantly higher for conidial mass of strain obtained from the insect. By day 5-7 of growth, the difference in diameters of colonies was $\Delta d = 0.7 \pm 0.1$ cm. The passaging method is aimed at maintaining pathogenic properties and increasing the virulence of the hyphomycete by means of proteins, chitin, and other biologically active substances contained in the insects. The method of passaging entomopathogenic fungus through organism of spore- (conidia-) infected insect allows to maintain aggressiveness of the *Beauveria bassiana* strain in laboratory conditions.

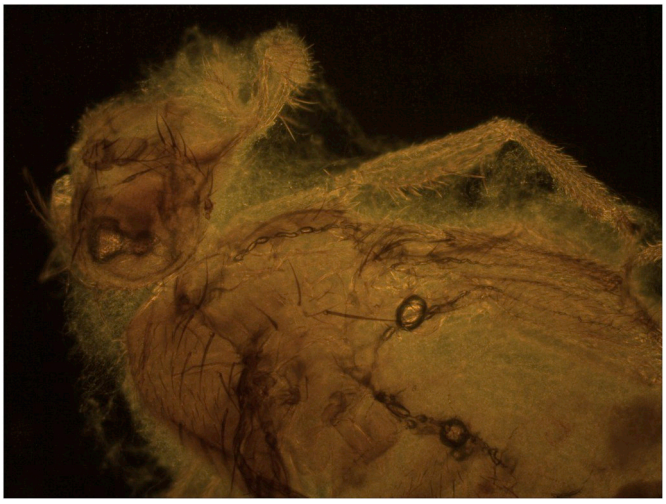

Fig. 2. Different mycosis-affected body parts of the *Drosophila melanogaster* fly.

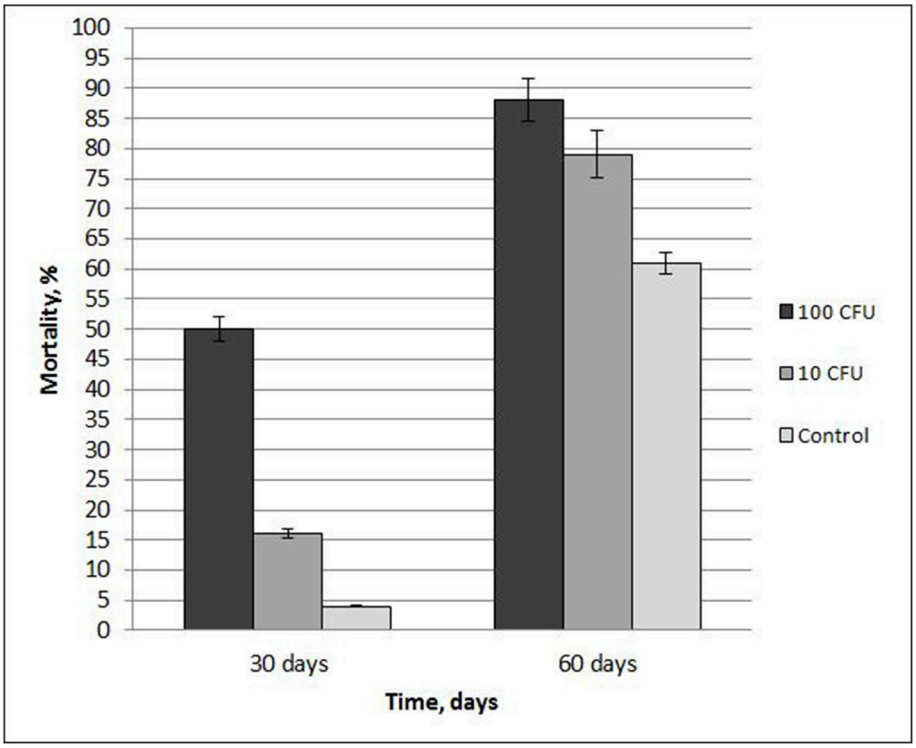

Fig. 1. Mortality of flies *Drosophila melanogaster,* treated with dry conidial mass of *Beauveria bassiana* strain.

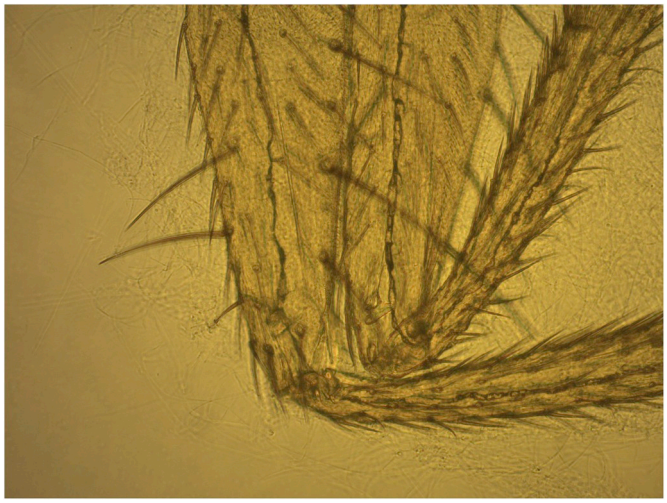

Fig. 3. Different mycosis-affected body parts of the *Drosophila melanogaster* fly.

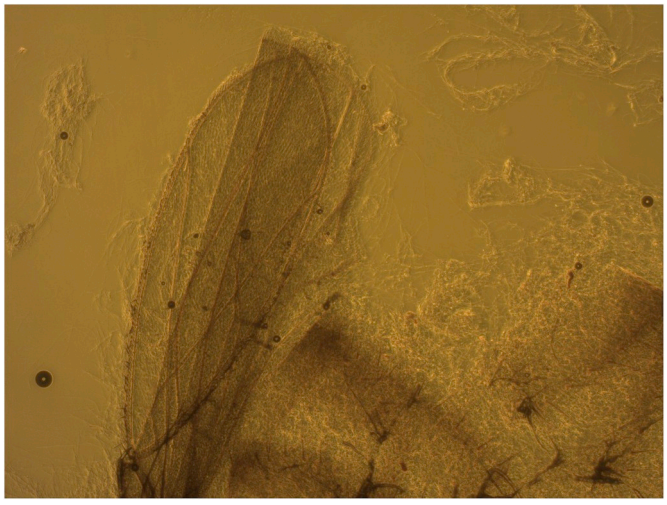

Fig. 4. Different mycosis-affected body parts of the *Drosophila melanogaster* fly.

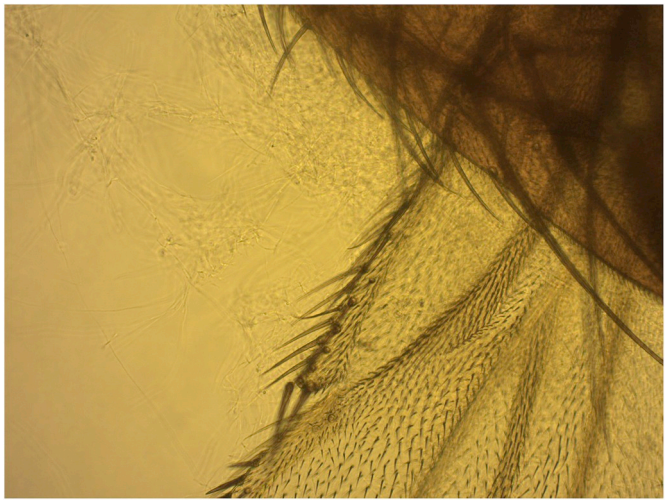

Fig. 5. Different mycosis-affected body parts of the *Drosophila melanogaster* fly.

3.3. Nematocidal activity of *Beauveria bassiana* strain

The test-organism for the study of nematocidal activity was selected due to the fact that free-living bacterial-feeding nematodes of the Rhabditidae family are morphologically different in the part that allows phytohelminths to parasitize (Masler et al., 1999; Chitwood, 1999; Zinovyeva et al., 2012). Depending on taxonomic position and way of life, some cuticle layers of nematodes may differ in thickness and ultrastructure. However, all nematode species have dermo -muscular body walls where proteins (keratin and collagen) determine elasticity and strength of coverings and lipoproteins ensure permeability (Zinovyeva et al., 2012). Therefore, agents which can inhibit nematode's mobility and even kill it when in contact with the nematode's body, have a toxic effect. The extent of such effect characterizes nematocidal activity and depends on the duration of contact and concentration of toxins.

Toxic effects on the test-organism were assessed based on mortality and changes in motility of nematodes (Table 2). The test-organism was treated with various types of liquid-phase medium in native form of preparations based on *Beauveria bassiana* strain. As a result, a dependency between concentration of culture suspension and its biological (nematocidal) activity towards motile stages of nematodes was found ($p \leq 0.05$; $r = 0.72$). When diluted suspension was used, biological activity decreased significantly, which was confirmed by nematostatic effect. It was noted that long incubation in water-based medium without toxins led to the death of some number of nematodes (natural losses – 2.5%). Toxicity of the native form of biopreparation (culture suspension without dilution) was observed for the suspension obtained from mixed medium with milk whey and spent grain liquor, that yielded 100% death of. Mortality of nematodes when using undiluted suspension of fungus on Czapek medium was at the level of 30%. This means that secondary metabolites of suspension, first of all their own (inherent to representatives of this fungi group) toxins of the *B.bassiana* strain, are active against motile stages of nematodes. Native form of the preparation as suspension of spore and mycelial fungal mass contains residual components of growth medium, which accelerates development of the pathological process. Probably, presence of Tween-80 inducer in the medium intensifies the toxic effect of fungus suspension on motile stages of nematodes. The semisynthetic Czapek medium is less favorable for synthesis of toxins, enzymes, and secondary metabolites of entomopathogenic fungus strain with nematocidal activity. Therefore, strain of *Beauveria bassiana* in certain conditions is able to produce enzymes and secondary metabolites in quantities sufficient to kill 90%

Table 2
Nematocidal activity of *Beauveria bassiana* strain-based biopreparations.

Treatment variant	Viability of mobile nematode stages, %	
	Incubation period	
	1st day (18 h)	2nd day (42 h)
Control (sterile water)	100% viable	2.5% mortality
Czapek medium-based suspension (undiluted)	6.7% mortality, decreasing activity	29.6% mortality, decreasing activity
Filtrated Czapek medium-based suspension (diluted 1:100)	3.3% mortality, decreasing activity	9.2% mortality, decreasing activity
Milk whey-spent liquor mixture-based suspension (undiluted)	91.6% mortality	100% mortality
Filtrated milk whey-spent liquor mixture-based suspension (diluted 1:100)	8.3% mortality, decreasing activity	10% mortality, decreasing activity

Note: Differences between control and experiment are reliable with $p \leq 0.05$.

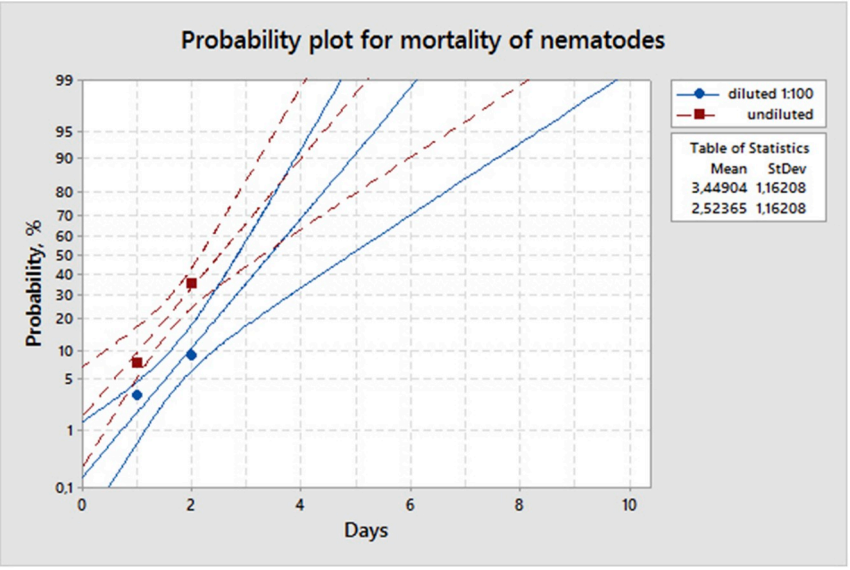

Fig. 6. Toxic effect of fungus suspension on motile stages of nematodes: Czapek medium-based suspension (undiluted and diluted 1:100).

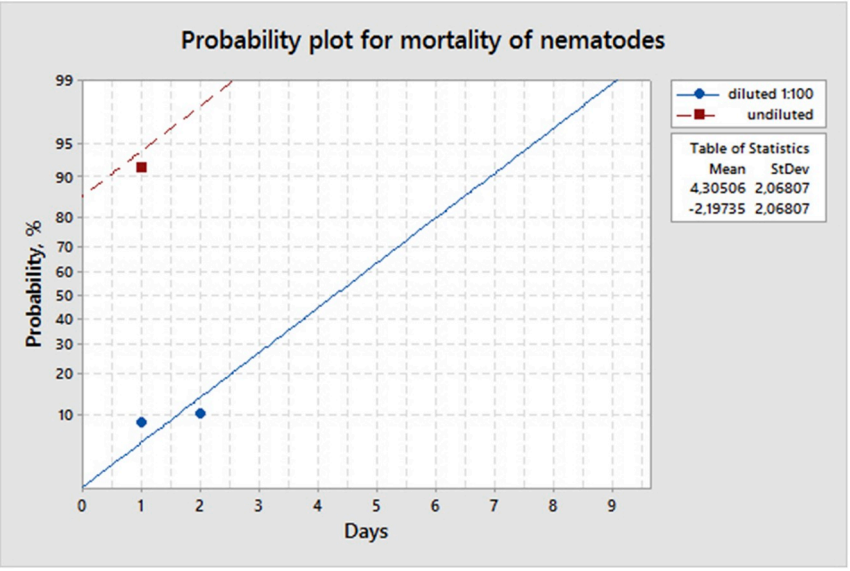

Fig. 7. Toxic effect of fungus suspension on motile stages of nematodes: Milk whey-spent liquor mixture-based suspension (undiluted and diluted 1:100).

of motile stages of nematodes (LC90). Results of the analysis of variance mostly confirm conclusions made based on simple comparison of empirical data (Table 2 and Figs. 6 and 7).

3.4. Carrier-immobilized biopreparation form

Mycelial mass and secondary metabolites from culture suspension based on *Beauveria bassiana* strain immobilized on brewer spent grain which contains grain hulls, fiber and other biodegradable substances. Due to the fact that drying and storage stage significantly reduces the original titer concentration of infectious units of the suspension, viability of carrier-immobilized form of the biopreparation has been evaluated (Table 3 and Figs. 8 and 9). After wetting, infectious units of *B.bassiana* strain immobilized on spent grain milled and dried at max. 50 ^{0}C have demonstrated high recovery rate and viability. High-temperature pre-treatment of brewer's spent grain was less favorable for

Table 3
Viability of immobilized biopreparation form.

Variant	Content of infection units, CFUs/g	
	before wetting (0 days)	after wetting (5 days)
1. Brewer spent grain (dried at 105 ± 5^{0}C)	(1.2 ± 0.2)x10^4	(2.6 ± 0.5)x10^6
2. Brewer spent grain (milled and dried at 45 ± 5^{0}C)	(9.6 ± 0.5)x10^4 (foreign microbiota: < 10^2)	(1.7 ± 0.5)x10^8 (foreign microbiota: < 10^2)

Fig. 8. Carrier-immobilized bio-preparation form, with differently treated brewery spent grain as carrier.

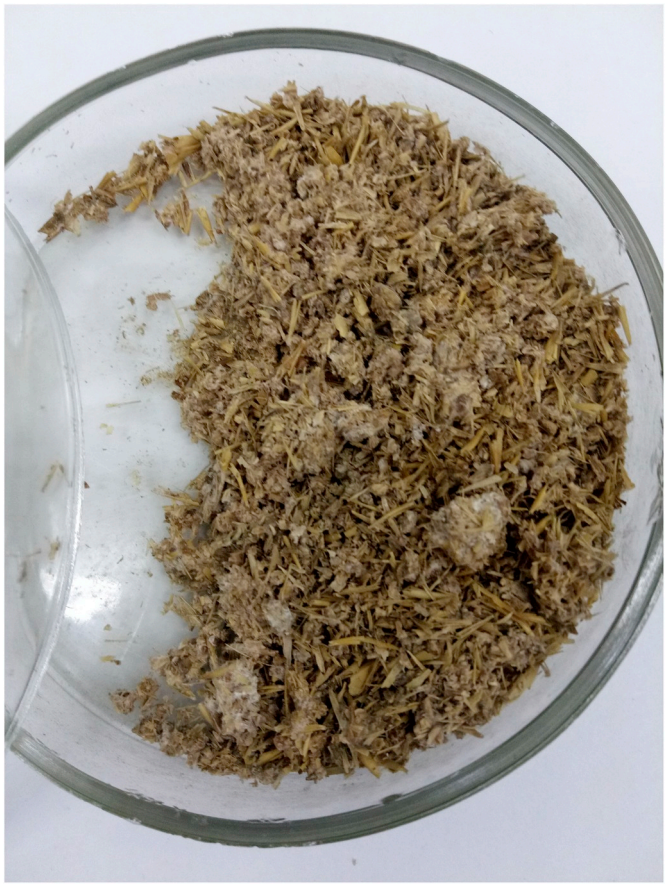

Fig. 9. Carrier-immobilized bio-preparation form, with differently treated brewery spent grain as carrier.

recovery and multiplication of the fungus on this carrier. Degradability of pulp contained in the spent grain is ensured by cellulolytic enzymes found in culture suspension of *Beauveria bassiana* strain (Table 1). The biopreparation in this form is degradable both by indigenous microbiota of the soil, and by biological component of the pesticide itself.

4. Conclusions

Results of the study allowed us to empirically confirm the prospects of using *Beauveria bassiana* entomopathogenic fungi strain to develop a biopesticide that features, among other characteristics, nematocidal activity. Native and immobilized forms of the biopreparation have been proposed that allow for different possibilities and conditions of its use for biological control of various phytophages.

Use of brewing and dairy production by-products was found to be promising to obtain different forms of biopreparation. Cultivation on suitable nutrition medium based on milk whey and brewing spent grain liquor mixture provides for high productivity and bio-synthesis of enzymes, apart from those enzymes that evolutionally constitute infectivity of entomopathogenic fungi. High catalase and cellulose-decomposing activity of the *Beauveria bassiana* strain was found.

The hyphomycete demonstrated insecticidal activity against the test-organism, particularly *Drosophila melanogaster* flies. Mortality rate was 50% with the infectious dose of 10^2 CFU. The authors proposed a method for passaging the *Beauveria bassiana* strain through insect's organism to maintain its pathogenicity and biological activity.

Nematocidal activity of the *Beauveria bassiana* strain towards the test-organism, particularly bacterial-feeding nematodes of the Rhabditidae family, was determined. It was found that secondary metabolites of suspension, primarily, own toxins of entomopathogenic fungus, demonstrate nematocidal activity against motile stages of nematodes. A dependency between biological (nematocidal) activity on concentration of suspension was determined. 90% mortality of nematodes was registered within 1 day of incubation when using native undiluted culture suspension of milk whey and brewer spent grain liquor with Tween-80 inducer.

A carrier-immobilized form of the biopreparation has been proposed where brewer spent grain was used as a biodegradable lignin-cellulose-containing carrier for biopreparation components (spore-containing mycelial mass and secondary metabolites). Brewer spent grain serves as additional source of nutrition and ensures activity and viability of the *Beauveria bassiana* strain.

Acknowledgements

This work was financially supported by the OOO 'AGROKHIM-BIOTEKH.

The author thanks colleagues from the Institute of Biology, Komi Science Centre, Alexei A. Kudrin and Svetlana N. Plusnina for their assistance.

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