TECHNOLOGY FOR OBTAINING COMPLEX MICROBIAL PREPARATION FOR BIOLOGICAL PROTECTION OF PLANTS FROM PESTS AND DISEASES USING BIOREACTOR

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Abstract. The article presents the results of the key technological stages involved in the production of a complex microbial preparation using a bioreactor. The study employed the bioreactor AF-0.170, designed for small-scale production of microbial pesticides and developed at the Engineering and Technological Institute "Biotechnika" during 2019-2020. The AF-0.170 bioreactor is intended for the submerged cultivation of microorganisms in liquid nutrient media, with agitation and aeration. The technological process includes several stages: bioreactor preparation; nutrient medium preparation; sterilization of the medium at 120 ºC for 120 minutes; medium inoculation with a microbial consortium consisting of *Pseudomonas aureofaciens* and *Streptomyces avermitilis* at 10% of medium volume; and cultivation of the complex microbial preparation for 48 hours at a temperature of 28 ºC, with a stirrer speed of 179 rpm and an aeration rate of 0.4 vvm. Under these technological conditions, a complex microbial preparation exhibiting both insecticidal and fungicidal activity was obtained, with a maximum titre of viable microbial cells reaching $5.0 \cdot 10^9$ CFU·cm^{-3}. The results of the experimental studies demonstrate that the fermentation equipment is suitable for scaling up production with only minor adjustments to the technological process. The combined use of biological agents produces a synergistic effect, enabling the simultaneous control of a broad spectrum of pests and diseases. A complex preparation for the biological protection of plants against pests and diseases, Diactaverm BT, has been developed. Diactaverm BT is a preparation based on the non-pathogenic soil actinomycete *Streptomyces avermitilis*, which is capable of producing biologically active substances (BAV) of various types and modes of action, including those with insecticidal properties, as well as the bacterium *Pseudomonas aureofaciens*, which possesses entomopathogenic activity and acts as an antagonist to many phytopathogens. The microbial insecticide-fungicide Diactaverm BT is used in both protected and open ground against aphids, mites, coleoptera, lepidopteran larvae and sawflies, apple fruit borers during the emergence of caterpillars, leaf rollers, and to reduce the virulence of phytopathogens causing fruit and root rot, moniliosis, fusariosis, scab and powdery mildew. The product obtained using this technology was tested under both controlled (greenhouse) and field conditions and demonstrated the following levels of efficacy: 90.5% against aphids, 80.0% against spider mites, 73.5% against pathogens causing root rot, and 57.1-89.4% against the main pests affecting plum crops.

Keywords: biological plant protection, complex microbial preparation, bioreactor, biosynthesis, verification.

Introduction

At present, unfortunately, the chemical method of plant protection continues to occupy a leading position among measures aimed at crop preservation. However, for many developed countries, the need to reduce the volume of pesticide use has become increasingly urgent, as these agents lack selectivity and therefore eliminate not only harmful organisms but also beneficial microorganisms and insects. Moreover, pesticides may enter food products, thereby reducing their quality. Their application is also associated with the adaptation of harmful species, leading to the emergence of pesticide-resistant forms within populations of pests and phytopathogens. Notably, the rate at which resistant forms develop outpaces the introduction of new chemical formulations. The necessity to limit the uncontrolled use of chemical pesticides in agricultural production technologies has prompted the search for alternative plant protection strategies. One such alternative, and a promising approach in this context, is biological plant protection [1-3]. Biological preparations based on living microorganisms and their metabolic products demonstrate significant potential for reducing the chemical burden on agrocenoses and increasing crop productivity. Their application results in agricultural produce free of pesticide residues and contributes to a reduction in environmental chemical load [4; 5]. In this context, complex preparations that combine the beneficial properties of several biological agents with different modes of action are of particular interest.

Complex preparations retain the advantages of plant protection products derived from monocultures while offering additional benefits. Their use contributes to reducing production costs and improving the quality of agricultural products, whereas the diverse range of biochemical compounds produced by biotechnological agents enhances physiological processes in plants. The synergistic interactions between carefully selected microbial producers result in an overall effect that exceeds the activity of individual strains.

The development of new, effective complex microbial preparations with fungicidal, insecticidal, and growth-promoting properties, aimed at increasing crop yields and improving product quality, constitutes a key area of both fundamental and applied research worldwide [6]. The creation of such multifunctional microbial preparations with different modes of action requires the development of appropriate production technologies.

The institute where the present research was conducted maintains a viable collection of industrially valuable microbial cultures for biologisation of agriculture, which represents a national asset [7]. On the basis of these collection cultures, complex preparations with various modes of action have been developed, studied, and introduced into production. The outcomes of the conducted research and development activities have led to the creation of several complex formulations, including: Trichopsin BT, characterized by enhanced fungicidal properties and partial insecticidal activity; Biodestructor BT, based on microbial consortia exhibiting cellulolytic and antagonistic functions; Vitastim BT and BioGibervit BT, which are complex natural plant growth regulators with fungicidal properties.

As a result of scientific research conducted between 2021 and 2025, the complex preparation Diactaverm BT was developed for biological protection of plants against pests and diseases. Diactaverm BT is based on the non-pathogenic soil actinomycete *Streptomyces avermitilis*, which belongs to a group of microorganisms capable of producing biologically active substances (BAS) with various mechanisms of action, including insecticidal effects, as well as the bacterium *Pseudomonas aureofaciens*, which exhibits entomopathogenic activity and antagonistic effects agaisnst numerous phytopathogens. The microbial insecticidal and fungicidal preparation Diactaverm BT is applied under both protected and open-field conditions to control a wide range of pests and diseases including aphids, mites, coleopteran insects, lepidopteran larvae, and sawflies; apple fruit borers during the larval (caterpillar) emergence period; and leaf rollers. The preparation also contributes to reducing the aggressiveness index of phytopathogens responsible for fruit and root rot, moniliosis, fusariosis, scab, and powdery mildew.

The development of complex preparations through the co-cultivation of different microorganisms represents a complex, multi-stage process, ranging from the screening of promising microbial strains to their practical implementation in production. The research findings indicate that the most promising combination for the development of a microbial complex preparation is the association of *Pseudomonas aureofaciens* and *Streptomyces avermitilis*, as it demonstrated the highest levels of fungicidal and insecticidal activity. Therefore, this microbial complex preparation was selected as the basis for development of the fermentation technology.

Materials and methods

The laboratory process for producing a microbial complex preparation involves the submerged cultivation of microorganisms in 3 dm³ glass vessels using the KPM 36/90 industrial microbiological shaker. The technological process includes the preparation of culture media, their thermal sterilisation in an autoclave followed by verification of microbiological purity, preparation of the inoculum under laboratory conditions, inoculation of the culture medium, and co-cultivation under selected operating conditions using the shaker.

Using this approach, a microbial complex preparation with a high total titre of viable microbial cells $7.0 \cdot 10^{10}$ CFU·cm^{-3} was obtained. However, the maximum production capacity of the KPM 36/90 microbiological shaker is limited to 54 dm^3 per cycle. Therefore, for large-scale production with minimal modifications to the technological process, the use of fermentation vessels (bioreactors) is considered appropriate. The main stages of the technological process for producing the complex preparation using a bioreactor have been developed. These include preparation of the bioreactor, preparation of the culture medium, sterilisation, verification of sterility, subculturing of the initial master culture in test tubes, propagation of the inoculum on Petri dishes, preparation of the inoculum in 3 dm³

glass vessels using the KPM 36/90 shaker, and inoculation of the culture medium in the fermenter. To determine the optimal process parameters required for the microorganisms included in the microbial consortium, biosynthesis was carried out under different mixing and aeration regimes.

A technology for producing the complex preparation in the bioreactor AF-0.170 has been developed. The process includes three main stages: pre-fermentation, fermentation, and post-fermentation.

Results and Discussion

1. Pre-fermentation stage

1. Preparation of the bioreactor

The fermentation vessel was washed with tap water, followed by disinfection of internal surfaces using cotton wool soaked in a 70% ethanol solution or a 5% hydrogen peroxide solution.

2. Preparation of the culture medium

A concentrated culture medium was prepared in a separate vessel and subsequently diluted with potable water directly in the fermentation vessel to 70% of its working volume. The concentrate consisted of a mixture of all medium components. Mineral salts were dissolved in a small volume of filtered tap water at a temperature of 40-50 ºC. The remaining components were added according to the formulation calculated per 1 dm^3 of the final culture medium.

The fermentation vessel was filled with 110 dm^3 of filtered tap water, followed by the addition of 10 dm^3 of the culture medium concentrate. The mixture was homogenised with the vessel closed using the fermenter stirrer at 109 rpm for 15 minutes. Subsequently, the pH of the culture medium was measured and adjusted to the optimal level, if necessary.

3. Sterilisation of the culture medium

The culture medium was sterilised directly in the fermentation vessel at a temperature of 120 ºC. Three exposure times were investigated: 60, 90, and 120 minutes.

4. Cooling of the culture medium

Cooling from the sterilisation temperature (120 ºC) to the inoculation temperature (28 ºC) was achieved through convective heat exchange with the surrounding air.

5. Verification of sterility

After cooling, the sterility of the culture medium was assessed by inoculation onto meat-peptone agar (MPA) and Sabouraud agar. Samples were incubated in a thermostat for 24 hours at 29 ºC (MPA) and for 72 hours at 27 ºC (Sabouraud agar). The absence of microbial growth confirmed the absence of contamination. Based on the results obtained for different sterilisation exposure times, 120 minutes was determined to be the optimal sterilisation duration.

2. Fermentation stage

Inoculation of the culture medium and cultivation conditions. The culture medium in the fermentation vessel was inoculated with a microbial consortium and cultivated under different mixing and aeration conditions.

The inoculum for the microbial complex preparation consisted of a bacterial culture of *Pseudomonas aureofaciens* and the actinomycete *Streptomyces avermitilis*. The inoculum was prepared using two methods:

- using the KPM 36/90 industrial microbiological shaker (standard technology);
- by washing cells with sterile water from Petri dishes inoculated with starter cultures.

The fermentation cycle for inocula obtained by both methods lasted 72 hours, with the medium temperature maintained at 28 ºC. The inoculum volume was 12 dm^3, corresponding to 10% of the total culture medium volume.

Intermediate assessments of microbial cell concentration and contamination of the culture medium with foreign microflora were performed every 24 hours by sampling.

Three mixing and aeration regimes were investigated for cultivation of the microbial complex preparation obtained using different inoculation methods (variants 1-6). The stirrer speed ranged from 179 to 220 rpm, while the aeration rate (defined as the volumetric air flow per unit volume of the fermentation medium) ranged from 0.2 to 0.4 vvm. The results of the experimental studies are presented in Tables 1 and 2.

Table 1

Results of cultivation of the complex preparation in the AF-0.170 bioreactor using inoculum obtained from shaking flasks

No	Initial titre of the culture, CFU·cm^{-3}	Mixer speed, rpm	Aeration rate, vvm	Cultivation time, hours	Level of contamination, %	Titer obtained, CFU·cm^{-3}
Option 1						
1	$6.4 \cdot 10^{10}$	220	0.2	24	0.4	$1.8 \cdot 10^{7}$
2	$6.4 \cdot 10^{10}$	220	0.2	48	0.7	$2.6 \cdot 10^{8}$
3	$6.4 \cdot 10^{10}$	220	0.2	72	0.9	$2.3 \cdot 10^{8}$
Option 2						
1	$5.8 \cdot 10^{10}$	210	0.3	24	0.5	$2.0 \cdot 10^{8}$
2	$5.8 \cdot 10^{10}$	210	0.3	48	0.9	$3.3 \cdot 10^{9}$
3	$5.8 \cdot 10^{10}$	210	0.3	72	1.0	$3.1 \cdot 10^{9}$
Option 3						
1	$5.6 \cdot 10^{10}$	179	0.4	24	0.4	$2.2 \cdot 10^{8}$
2	$5.6 \cdot 10^{10}$	179	0.4	48	0.9	$5.0 \cdot 10^{9}$
3	$5.6 \cdot 10^{10}$	179	0.4	72	1.2	$3.0 \cdot 10^{9}$

Table 2

Results of culturing the complex preparation in the AF-0.170 fermenter using an inoculum obtained by scraping cultures from Petri dishes

No	Initial titre of the culture, CFU·(cm^3)$^{-1}$	Mixer speed, rpm	Aeration rate, vvm	Cultivation time, hours	Level of contamination, %	Titer obtained, CFU·cm^{-3}
Option 4						
1	$5.5 \cdot 10^{10}$	220	0.2	24	0.2	$5.6 \cdot 10^{7}$
2	$5.5 \cdot 10^{10}$	220	0.2	48	0.3	$7.8 \cdot 10^{8}$
3	$5.5 \cdot 10^{10}$	220	0.2	72	0.3	$7.3 \cdot 10^{8}$
Option 5						
1	$5.3 \cdot 10^{10}$	210	0.3	24	0.2	$2.2 \cdot 10^{8}$
2	$5.3 \cdot 10^{10}$	210	0.3	48	0.3	$3.6 \cdot 10^{9}$
3	$5.3 \cdot 10^{10}$	210	0.3	72	0.3	$3.4 \cdot 10^{9}$
Option 6						
1	$5.0 \cdot 10^{10}$	179	0.4	24	0.2	$2.0 \cdot 10^{8}$
2	$5.0 \cdot 10^{10}$	179	0.4	48	0.3	$5.0 \cdot 10^{9}$
3	$5.0 \cdot 10^{10}$	179	0.4	72	0.3	$4.2 \cdot 10^{9}$

The results of verification of the microbial complex preparation as a function of incubation time in the bioreactor are shown in Fig. 1. The samples were inoculated onto MPA and Sabouraud agar.

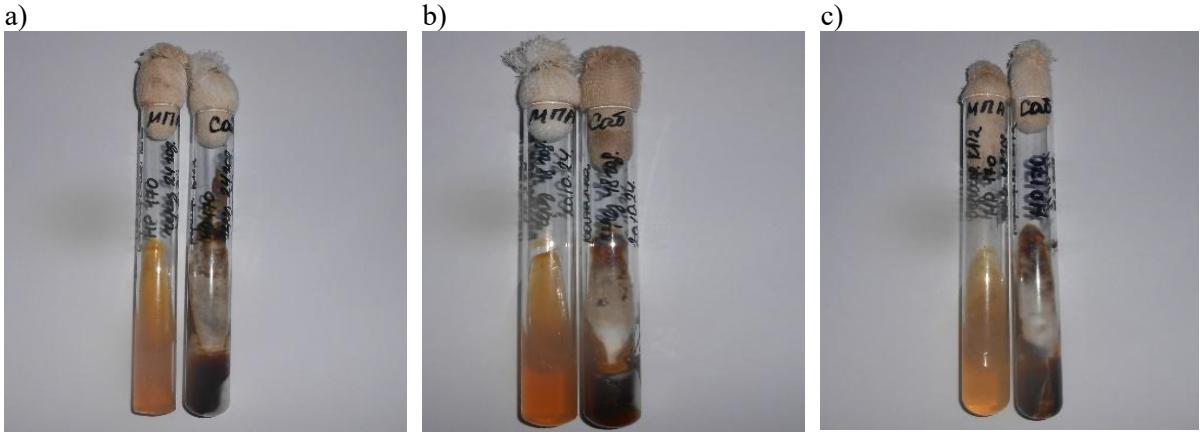

Fig. 1. **Results of verification of the microbial complex preparation as a function of incubation time for variant 6:** a – 24 h; b – 48 h; c – 72 h

The results of the study, with respect to cultivation time in the bioreactor, demonstrated that the highest concentration of viable microbial cells was achieved after 48 hours of cultivation, whereas after 72 hours the titres remained nearly unchanged (Table 2). However, *Pseudomonas aureofaciens* lost its ability to produce biologically active substances of the phenazine carboxylic acid class, as indicated by the change in colony colour from orange to white. Therefore, 48 hours can be considered the optimal cultivation time.

3. Post-fermentation stage

The post-fermentation stage includes filling the complex preparation into PET containers, washing the fermentation vessel, and subsequent sterilisation.

As a result of the conducted research, a technology for producing a complex microbial preparation using the bioreactor AF-0.170 was developed. The technological process includes preparation of the bioreactor, preparation of the culture medium according to the formulation, its sterilisation at a temperature of 120 ºC for 120 minutes, inoculation of the AF-0.170 fermenter with a consortium of microorganisms (*Pseudomonas aureofaciens* and *Streptomyces avermitilis*) at 10% of the culture medium volume, and cultivation for 48 hours (Fig. 1) at a temperature of 28 ºC, with a stirrer speed of 179 rpm and an aeration rate of 0.4 vvm. Under these technological conditions, a complex preparation with insecticidal and fungicidal activity was obtained, with a maximum titre of viable microbial cells of $5.0 \cdot 10^9$ CFU·cm^{-3}. The product obtained using this technology was tested under controlled conditions and demonstrated the following levels of efficacy: 90.5% against aphids, 80.0% against spider mites, 73.5% against pathogens causing root rot, and 57.1-89.4% against the main pests of plum crops. In recent years, particular attention has been paid to the development of complex microbial preparations consisting of combinations of compatible microbial strains with different mechanisms of antagonistic activity. The use of microbial associations significantly enhances the effectiveness of biocontrol agents due to synergistic interactions between components, expansion of the spectrum of antagonistic activity, and increased resistance to environmental variability. In study [8], it is demonstrated that combined inoculants based on Bacillus subtilis, Trichoderma harzianum, and Pseudomonas fluorescens exhibit a more pronounced protective effect compared to monocultures. This effect is attributed to the combination of different biocontrol mechanisms, including competition for substrates, synthesis of antibiotic compounds, and induction of systemic plant resistance.

The positive effect of microbial consortia on soil has been demonstrated in [9]. The study showed that the introduced microorganisms did not significantly affect the metabolic diversity of the soil microbial community; however, they altered the utilisation of carbohydrates, complex carbon compounds, and organic phosphorus-containing compounds.

One of the key factors determining the effectiveness of microbial preparations is the development of technologies for their industrial production using fermentation equipment. Fermentation technologies

make it possible to ensure a high concentration of viable microbial cells, maintain the stability of the physiological state of microbial cultures, and guarantee the reproducibility of process parameters. In this context, particular importance is attached to optimising the composition of culture media, cultivation parameters, and the conditions required for the co-cultivation of microorganisms within microbial consortia. The authors of [10] demonstrate that the successful application of fermentation technology depends on the optimisation of multiple factors and the use of appropriate systems to ensure the production of stable and high-quality microbial inoculants.

The process of biopesticide synthesis comprises multiple stages, ranging from the isolation of microorganisms from diverse natural habitats to the evaluation of their activity against various pathogens. To fully exploit the potential of promising microbial strains, optimization of the biopesticide production process constitutes a critical step. Parameters such as hydrogen ion concentration (pH), temperature, and inoculum volume are of particular importance, especially in the large-scale production of secondary metabolites or enzymes. As demonstrated by the authors in [11], biopesticide production is highly dependent on the physicochemical conditions of scale-up. A thorough understanding of the influence of key parameters – such as temperature, carbon and nitrogen sources, aeration, and pH – facilitates more efficient and sustainable biopesticide production.

Conclusions

1. The experimental results demonstrated that, for large-scale production with minimal modification of manufacturing processes, the use of fermentation vessels is advisable.
2. The effective combination of biological agents produces a synergistic effect, enabling the simultaneous control of a wide range of pests and diseases.
3. The preparation produced using this technology has been tested under both greenhouse and field conditions and demonstrated the following levels of efficacy: 90.5% against aphids, 80.0% against spider mites, 73.5% against pathogens causing root rot, and 57.1-89.4% against the main pests of plum crops.

Author contributions

Research, data analysis, and manuscript preparation: N.P; peer review and editing: V.Y., M.T., A.A, A.R., O.B., H.T. and O.T. All authors have reviewed and approved the final version of the manuscript.

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