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Biotechnology of Microorganisms Growing– Fundamentals for the Development of a Litter Biodestructor

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The purpose of the research work was to select the optimal conditions for the cultivation of microorganisms. As a result of the conducted research work, the modes of growing a nitrogen-fixing culture and a microorganism with high enzymatic activity were selected and worked out. At the same time, the optimal conditions for the cultivation of *Azotobacter sp* were determined – the temperature optimum for cell accumulation was 30°C, for increased polysaccharide production 20 °C, aeration within 5-10 l/l/min, agitator speed-150 rpm, pH value within 6.0±0.2 units, which allowed to achieve a cell titer of at least 1.0×10^9 CFU/ml. A cost-effective nutrient medium was

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selected for growing *Pseudomonas sp*. molasses-autolysate medium and optimal conditions for growing the culture: cultivation temperature 30-32 °C, aeration 1.0-1.5 l/l/ min, agitator speed 150-200 rpm, pH 6.8-7.2 units, sub-titration 5.0 % KOH, defoaming with adecanol, cultivation time-72 hours, which allowed to achieve a cell titer of at least 1.0×10^9 CFU/ml.

Keywords: Litter; destructor; microorganisms; cultivation; culture medium; titer; reducing sugars; dynamics; cultivation mode.

1. INTRODUCTION

Poultry farming is one of the key branches of agriculture in the world. In recent years, it has been actively developing, introducing innovations and new technologies. At the same time, the level of consumption of poultry products is high and continues to increase constantly, which confirms the prospects of the entire poultry industry for at least the next few years [1,2,3,4].

It should be noted that the existing wide network of large and small farms is experiencing problems with the processing of manure, due to the long period of its natural neutralization. The problem of waste disposal is relevant, since a large amount of arable land is used for their storage, and litter storage facilities are a source of unpleasant odors that spread over long distances. The Ministry of Natural Resources of the Russian Federation in 2002 approved the "Federal Classification catalog of waste", which, for example, includes chicken manure with its classification to the III and IV hazard classes. And taking into account the decree of the Government of Russia 2003 No. 344 for the placement of waste of class III-moderately dangerous (bird droppings), a fine is charged from poultry farms, which leads to the loss of a large amount of money [5,6-9].

In this regard, the issue of processing by-products, in particular manure, remains open, and the search for means of its disposal and further use as biofertilizers with high biological activity is relevant and promising.

2. MATERIALS AND METHODS

In the process of developing the preparation of the litter biodestructor and its production technology, a mixture of two cultures of microorganisms *Azotobacter sp*., which is part of the group of aerobic gram-negative bacteria fixing molecular nitrogen, and *Pseudomonas sp*., which has proteolytic activity, was used, and their cultivation modes were tested. When determining the optimal conditions for growing microorganisms, the composition of nutrient media was studied, the dynamics of cell titer growth (CFU) and the yield of a microbial product with increased biological activity were studied. The cultures were obtained from ATCC.

During the cultivation of *Azotobacter* cells, samples were taken under sterile conditions for periodic analysis and regulation of the fermentation process. In the selected samples, reducing substances (pB,%) were determined by the Bertrand titrometric method, the number of cells (CFU/ml) – by seeding on dense media (Koch method), the amount of polysaccharide (g/l) – by alcohol deposition, the active acidity index (pH) - by the potentiometric method. Microscopy of the selected material was used to determine the change in the cell capsule. During the fermentation of *Pseudomonas sp*. the main indicator determined is the number of cells (CFU/ml) – by seeding on dense media (Koch method). The total titer of microorganisms was determined by the number of grown colonies. The number of viable cells in 1.0 ml of the drug (X) was calculated by the formula (1):

$$X = N \times P, \quad (1)$$

N is the arithmetic mean of the number of colonies in Petri dishes;
P is the ordinal number of the tenfold dilution in which the growth of bacteria is noted.

The results of counting the number of microorganisms on nutrient media were carried out in three repetitions to obtain more reliable results.
Control of the process of obtaining the preparation-biodestructor was carried out continuously-from taking the museum culture to obtaining the final product of fermentation.

3. RESULTS AND DISCUSSION

Selecting microorganisms in the composition of the preparation for the biodegradation of poultry droppings, analyzing the range of the most suitable cultures of microorganisms for its

construction, we took into account the properties of the strains, the name and number of functional metabolites produced that stimulate the acceleration of the natural fermentation of poultry droppings, namely, reducing the level of ammonia nitrogen, the titer of Escherichia coli, suppression of putrefactive microflora, reducing helminth infection and, as a result, providing an increase in the environmental safety class of the litter.

In accordance with the functions of the cultures of microorganisms, we focused on such properties as nitrogen fixation, which affects the reduction of the level of ammonia nitrogen, as well as proteolytic activity, which promotes the cleavage, biodegradation of protein into simpler organic compounds, which, being in a more accessible form for the native microflora, provide the best technological effect [10,11,12-13].

Based on the monitoring of existing preparations and cultures of microorganisms with the properties of interest to us, such cultures of microorganisms as *Azotobacter sp* were selected. - nitrogen bioutilization agent and *Pseudomonas sp*., which is a destructor of aromatic compounds.

3.1 Cultivation of *Azotobacter sp.*

Culture of *Azotobacter sp*. it is a member of the group of aerobic gram-negative bacteria that fix molecular nitrogen. Young cells of this culture are short, thickened rods with a thin capsule, which is sometimes absent, which is why the cocci are quite close to each other, representing paired (diplococci) and tetroid (tetrococci) formations. As they age, they lose their mobility and become covered with a common capsule. In liquid media with good aeration, the development time of the azotobacter is reduced to several days.

The time of passage of the development cycle of Azotobacter cells varies and depends on the conditions of their cultivation. It is known that on solid media, azotobacter develops within a week or more, while on liquid media with good aeration, the development time of azotobacter is reduced to several days [14, 15].

Preparation of a drug based on *Azotobacter sp*. involves the storage of microorganisms in the museum of pure cultures on the Burc medium, the production of the mother culture on the Ashby medium, the production of the seed culture on the modified Ashby medium, further sowing at a dose of 10.0% by weight of the nutrient medium in the fermentation unit "Oka MF-100" (Fig. 1), designed for the implementation of the processes of cultivation of microorganisms, biosynthesis, biocatalysis and biotransformation of biologically active substances using energy-saving technology with the use of bacteria, yeast, mycelial bacteria, fungi, microalgae, and tissues as producers. The plant has a set of computer programs that provide the modes of periodic and continuous cultivation of microorganisms.

Fig. 1. Fermentation equipment "Oka MF-100"

Cultivation was carried out under conditions of strict purity of the culture, which is associated with the sterilization of both the main and auxiliary equipment, as well as all components of the medium entering the fermenter.

The continuous fermentation procedure itself was carried out according to the following algorithm: loading the medium from the initial container into the fermenter, sterilization, cooling, inoculate seeding through the upper steam-sterilized connector, turning on the aeration air, temperature control systems, pulsators, and monitoring the process by sampling through the lower steam-sterilized connector.

Storage of museum culture *Azotobacter sp*. the following composition was carried out on a dense Burc nutrient medium (g/l distilled water): mannitol – 10.0; K_2HPO_4 – 0,64; NaH_2PO_4 – 0,16; NaCl – 0,2; $MgSO_4 \times 7H_2O$ – 0,2; $CaCl_2$ – 0,1; with the following trace elements (mg/l): $FeSO_4 \times 7\ H_2O$ – 2,5; H_3BO_3 – 2.9; $CoSO_4 \times 7\ H_2O$ – 1,2; $CuSO_4 \times 7\ H_2O$ – 0,1; $MnCl_2 \times 4\ H_2O$ – 0,09; $Na_2MoO_4 \times 2\ H_2O$ – 2,5; $ZnSO_4 \times 7\ H_2O$ – 1,2.

Active acidity for optimal growth of *Azotobacter sp*. it was kept within the range of 7.0±0.2 at the initial stage of growth (0-6 hours), and from 12 hours of cultivation, the pH was maintained at about 6.0±0.2. To maintain the acidity within these limits, a 5.0% KOH solution was used as a titrant.

The mother culture of *Azotobacter sp*. prepared on Ashby medium of the following composition (g/l distilled water): K_2HP0_4 – 0,2; $MgS0_4 \times 7H_20$ – 0,2; NaCl – 0,2; KH_2P0_4 – 0,1; $CaCO_3$ – 5,0; mannitol (or sucrose) – 20.0.

The seed culture was prepared similarly to the mother culture, but with the replacement of mannitol with corn molasses in order to reduce the cost of finished products (modified Ashby medium). It was found that the replacement of this component of the Ashby culture medium did not lead to a change in the qualitative and quantitative parameters of the culture under study, since the titer of the culture was 2.3×10^8 CFU/ml. Fermentation continued for 72 hours. For periodic analysis and regulation of the fermentation process, samples were taken under sterile conditions.

The amount of polysaccharides in the cultivation process is an important indicator, since the polysaccharide of the protective capsule provides the best preservation of the biomass during storage, is one of the main sources of energy. Its formation occurs during the cultivation of the crop, the amount depends on the cultivation mode, mainly on the temperature. In addition, the polysaccharide is not only in the capsule, but also released into the external environment, which causes a high viscosity of the culture liquid after cultivation [16-23]. It is also necessary to take into account the ability of microbial polysaccharides to adsorb toxic metabolites in the gastrointestinal tract of farm animals when using the culture as part of feed additives [24-28,29].

In the process of cultivation of *Azotobacter sp*. there is an increase in the synthesis of polysaccharide, its amount for 72 hours of fermentation was 9.7 g/l. This is due to the fact that when cells get into extreme conditions, they accumulate a large amount of polysaccharide, forming protective capsules, and also release it into the medium, which is due to the increased viscosity of the culture liquid. It is the amount of polysaccharide that affects the increase in the shelf life of the culture liquid, and subsequently of the biological product. Dynamics of changes in the amount of polysaccharide during the cultivation of *Azotobacter sp*. shown in Fig. 2.

The amount of reducing substances in the cultivation process is reduced by 76% and by 72 hours of fermentation is 0.6 %, which is caused by the introduction of cell culture in "extreme" conditions for it. Cells with a sharp decrease in the culture temperature from 30 to 20°C, begin active consumption of reducing substances, in order to increase the protective capsule of the cell [30]. The changes in the amount of reducing substances during cultivation are shown in Fig. 3.

During the entire period of cultivation, the number of cells of the studied culture of the microorganism was determined. It was found that the cell titer increased and by the end of fermentation was 2.1×10^9 CFU/ml, and a sharp decrease in temperature did not affect the cell death, which is associated with the presence of reducing substances that are a source of energy for the cell.

As a result of a series of successive fermentations, the optimal conditions for the cultivation of *Azotobacter sp* were determined. The temperature optimum for cell accumulation was 30 °C, for increased polysaccharide

production 20 °C, aeration within 5-10 l/l/min, agitator speed-150 rpm, the pH value was maintained within 6.0±0.2.

3.2 Cultivation of *Pseudomonas sp.*

The second culture included in the preparation of the poultry litter bioutilizer was *Pseudomonas sp.*, which is a mobile small sticks 0.5-1.0×1.5-5.0 microns arranged singly, in pairs, very rarely in the form of a chain of 3-4 cells, Gram-positive, spores and capsules do not form. Growth limits + 10°C–+35°C.

This microorganism is an active producer of a highly active proteolytic complex. The culture is not pathogenic, virulent, refers to saprophytes (microorganisms living in natural, natural conditions and taking part in the decomposition of organic residues).

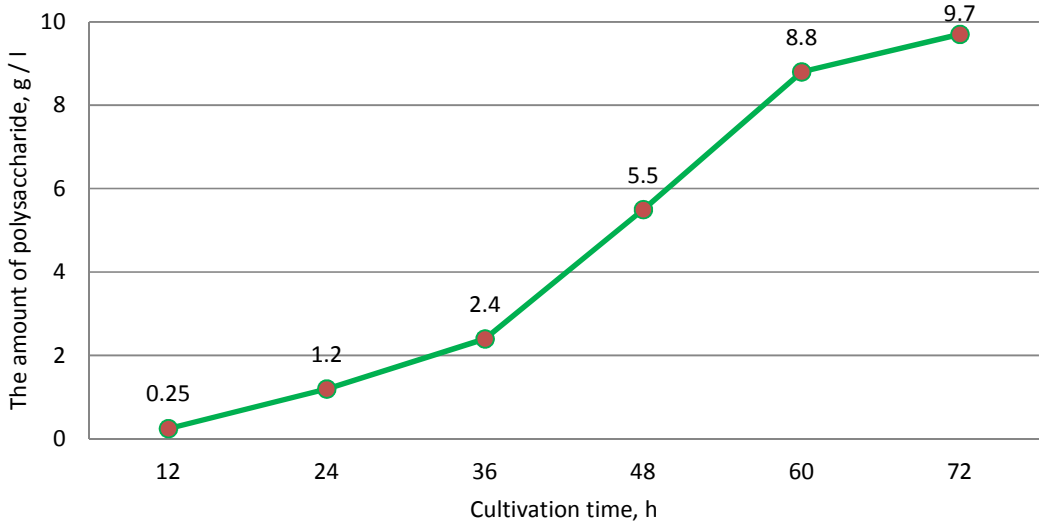

Fig. 2. Changes in the amount of polysaccharide during the cultivation of *Azotobacter sp*

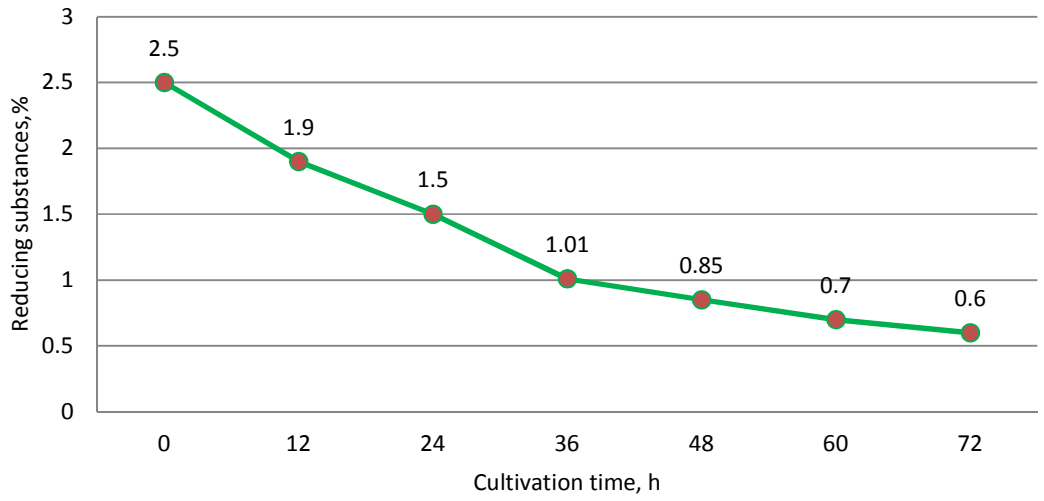

Fig. 3. Changes in the amount of reducing substances during the cultivation of *Azotobacter sp*

The selection and analysis of the composition of the most common nutrient media for the cultivation of *Pseudomonas sp*. in 250 ml Erlenmeyer flasks on an orbital temperature-controlled shaker. The following nutrient media were used:

– culture medium LB;
– King V nutrient Medium;
– glucose-peptone medium (Golubev medium);
– molasses-autolysate (MA) nutrient medium

Since the synthesis of some enzymes, in particular protease, as well as many secondary metabolites is subject to nitrogen repression, the production of these compounds can be increased as a result of replacing ammonium in the nutrient medium with less efficient sources of nitrogen.

Often, protease preparations contain a whole complex of enzymes with different properties and simultaneously complexes of enzymes with similar physicochemical and catalytic properties (isoenzymes). The isoenzymes are identical in their catalytic action, but differ in their biophysical constants. The biosynthesis of enzymes by microorganisms is closely related to the main conditions affecting the growth and development of crops, and primarily to the composition of the nutrient medium. The sources of nitrogen and carbon in the nutrient medium affect both the constructive exchange of cultures and the synthesis of enzymes. There are different opinions about the influence of various sources of nitrogen nutrition in the environment on the growth of microorganisms and the formation of proteolytic enzymes. Some authors believe that proteins are the only favorable source of nitrogen for better growth of microorganisms and enzyme synthesis, while others argue that a combination of mineral and protein substrates should be used as the best source of nitrogen, and finally, a number of authors believe that only mineral salts can be the only source of nitrogen [24,31].

The well-known culture media LB and King B, which are the main ones for the cultivation of bacteria of the genus *Pseudomonas sp*., have a disadvantage – high cost, since it contains such an expensive component as peptone. The use of a glucose-peptone medium (GPM) at the first stage was due to the versatility of this medium and the good growth of *Pseudomonas sp* bacteria. The achieved titer of the cells of the studied culture when grown on this medium on rocking flasks was 10^7-10^8 CFU/ml.

In the industrial production of biological products based on Pseudomonas bacteria intended for use in microbiological production, the high cost of the nutrient medium will inevitably lead to an increase in the price of the target product.

One of the tasks set was to reduce the cost of the culture medium for the cultivation of Pseudomonas bacteria and to increase the yield of biomass with the presence of a proteolytic enzyme complex.

The technical task of reducing the cost of the nutrient medium and increasing the yield of biomass was solved by using a molasses-autolysate (MA) nutrient medium, which includes corn molasses as a carbon source, which is a waste product and contains up to 45.0 % sucrose and other reducing sugars (Table 1). Later, this medium was used as the basis of the production environment for the production of *Pseudomonas sp* culture.

Preparation of the drug based on *Pseudomonas sp*. involves the storage of microorganisms in the museum of pure cultures on agarized medium King B, the production of masterbatch cultures on glucose-peptone medium of Golubev, the production of seed culture on molasses-autolysate medium (MA), further sowing at a dose of 10.0% by weight of the nutrient medium MA in the fermentation unit "Oka MF-100".

The following mode of cultivation of masterbatch and seed culture on a thermostatically controlled orbital shaker was selected: constant aeration-200 rpm, cultivation temperature-30 °C, cultivation time-72 hours. The cell titer at the end of fermentation is 1.0×10^8 CFU/ml.

After testing the cultivation modes at the Oka MF-100 fermentation plant, the optimal values of these parameters were determined in terms of aeration, temperature and time modes. For the culture of *Pseudomonas sp*. on the molasses-autolysate medium, the optimal growing conditions were: cultivation temperature 30-32 °C, aeration 1.0-1.5 l/l / min, agitator speed 150-200 rpm, pH 6.8-7.2 units, sub-titration 5.0% KOH, defoaming with adecanol, cultivation time-72 hours. The achieved cell titer was 1.1×10^9 CFU / ml.

In the cultivation of *Pseudomonas sp*. in the fermenter "OKA MF-100", it was noted that the results of the selection of cultivation modes showed good repeatability and allows us to talk

about the possibility of further scaling of fermentation processes in larger plants (from 1000 liters and more), which is important when obtaining this component of the biological product in industrial conditions.

The effect of the drug is based both on the further development of the culture at the object of application, and on the direct action of the culture liquid with enzymes.

After obtaining the cell biomass with a given titer in the fermenter, the culture liquid with the cells is aseptically packaged in prepared aseptic containers. Before using the biological product, storage is carried out at a temperature of + 5^{o}C.

3.3 Technology for Obtaining a Component Preparation-biodestructor

The technology for obtaining a drug-a biodestructor of manure includes the following main stages of production: storage in the museum of pure cultures, production of masterbatch and seed culture, fermentation at the fermentation plant *Azotobacter sp*. and *Pseudomonas sp*., mixing of cultures of microorganisms in a certain ratio, packaging and storage (Fig. 4).

Storage of *Azotobacter sp*. in the Museum of pure Cultures, it is carried out on a nutrient agarized Burc medium. In the pre-prepared test tubes, a sterile medium is poured and left to completely solidify at an angle of 45°, after checking for sterility in a laminar box in the sterile zone of the burner flame, a stroke was seeded using a microscopic loop. The test tubes are sent to a thermostat with a temperature of 26-28 °C for 5 days. Test tubes with grown culture of *Azotobacter sp*. without the presence of foreign microorganisms, they were stored at a temperature of 2-4°C for 4-6 months until the next re-sowing process.

Table 1. Comparative cost estimation of the components of the studied media

Medium	Components	Reagent consumption per 1 liter of medium, kg / l	The cost of reagents per 1 kg, RUR	Cost per 1 liter of medium, RUR
LB	Peptone (enzymatic)	0.010	2304.73	23.05
	Yeast Extract	0.05	2665.38	133.27
	NaCl	0.010	118.0	1.18
	Total. RUR.			157.4
King B	Peptone	0.020	2304.73	46.09
	Glycerol	0.010	352.98	3.53
	K_2HPO_4	0.0015	649.37	0.97
	$MgSO_4x7H_2O$	0.0015	152.45	0.23
	Total. RUR			50.82
Golubev's glucose-peptone medium	Na_2HPO_4	0.0032	262.99	0.84
	K_2HPO_4	0.0003	649.37	1.95
	$MgSO_4x7H_2O$	0.0005	152.45	0.08
	NaCl	0.0005	118.0	0.06
	Peptone (enzymatic)	0.002	2304.73	4.61
	Yeast Extract	0.0005	2665.38	1.33
	Glucose	0.025	262.45	6.56
	Total. RUR.			15.43
Molasses-autolysate (MA) nutrient medium	Corn molasses	0.045	4.0	0.18
	K_2HPO_4	0.002	649.37	1.30
	$MgSO_4x7H_2O$	0.0015	152.45	0.23
	Yeast autolysate	0.000268	11594.39	3.11
	Total. RUR			4.82

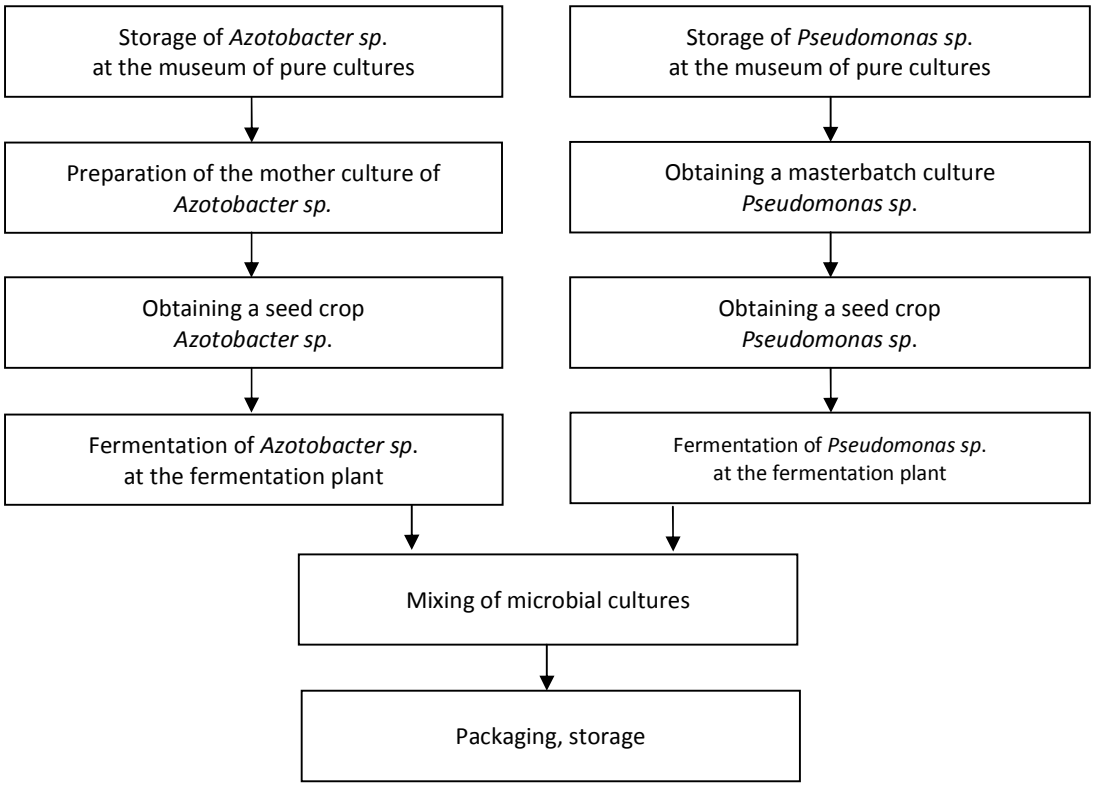

Fig. 4. Scheme of preparation of the drug-biodestructor

The mother culture of *Azotobacter sp*. prepared on Ashby liquid medium. The prepared medium was poured into flasks and autoclaved at 1 atm. for 40 minutes. After holding the medium to determine the sterility, it was seeded with 2.0 ml of the test culture. For this purpose, in a laminar box in the sterile zone of the burner flame, using a dispenser, sterile water and a microbiological loop, colonies were flushed from the beveled agarized medium into flasks with Ashby medium. The seeded flasks were installed on a thermostatically controlled rotary shaker, the rotation speed was-150-160 rpm, the temperature was 24-26 °C, the fermentation duration was 72 hours. At the end of the cultivation time, the resulting culture was evaluated visually and by microscopy. Storage at a temperature of + 2-4 °C. The seed culture was prepared similarly to the mother culture, but with the replacement of mannitol with corn molasses.

Storage of *Pseudomonas sp*. in the museum of pure cultures, it is carried out on agarized medium King V. The production of the mother culture was carried out using the glucose-peptone medium of Golubev, the production of the seed culture provided for the use of molasses-autolysate medium, the production of the working culture in the conditions of the fermentation plant also using the MA medium.

The preparation of the finished form of the drug involves mixing *Azotobacter sp*. and *Pseudomonas sp*. in a certain ratio, further packaging in a polymer sterilized container and storage of the drug.

4. CONCLUSION

Thus, to obtain a biological product with bioutilization properties, the cultivation modes of *Azotobacter sp* cultures were selected and tested. and *Pseudomonas sp* according to the specified biological parameters. Optimal conditions for the cultivation of *Azotobacter sp* were determined – the temperature optimum for cell accumulation was 30 °C, for increased polysaccharide production 20 °C, aeration within 5-10 l/l /min, agitator speed-150 rpm, pH value within 6.0±0.2 units, which allows to achieve a cell titer of at least 1.0×10^9 CFU/ml. A cost-effective nutrient medium was selected for

growing *Pseudomonas sp*. мел molasses-autolysate medium and optimal conditions for growing the culture: cultivation temperature 30-32 °C, aeration 1.0-1.5 l/l /min, agitator speed 150-200 rpm, pH 6.8-7.2 units, sub-titration 5.0 % KOH, defoaming with adecanol, cultivation time-72 hours, which allows you to achieve a cell titer of at least 1.0×10^9 CFU/ml.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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