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Methionine, *Azotobacter chroococcum* Growth Activator

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Abstract

A scheme of low-tonnage production of the microbial preparation based on the *Azotobacter chroococcum* VKM B-1616 strain intended for stimulating the growth and protecting of plants has been developed. It has been found that the nutrient medium containing sucrose and wheat bran and prepared according to the two-phase fractional scheme provides the same growth properties of *A. chroococcum* as the Beyerinck medium. Adding glycine to the medium increased the yield of microorganisms 2.5 times; lysine had no significant effect, and phenylalanine reduced the yield of microorganisms 3 times. Methionine had the greatest stimulating effect - the yield of microorganisms increased 8 times. The highest yield of microorganisms was in the medium that contained salts of molybdenum in addition to sucrose, bran, and methionine. With that, the number of cell divisions during the period of the logarithmic phase increased from 10 to 17.3, and the time of generation reduced from 7.3 to 2.4 hours. The scheme provides the possibility of producing comprehensive biological products for agricultural enterprises in the immediate vicinity of the location of their use.

Keywords: *Azotobacter chroococcum*, biofertilizers, low-tonnage microbial preparations' production, methionine, nitrogen-fixing activity.

INTRODUCTION

Efficient management of agrocoenoses involves not only maximizing the genetic potential of the crops, but maintaining high levels of soil fertility as well. This problem can only be solved with the use of, along with efficient cultivation methods and rational crop rotation, microbiological preparations that can ensure preservation of soil fertility, and plant protection.

The modern market of biological protection agents is presented by Russian preparations. Their share is 99.4% of the total market. Of these, insecticides account for 6.5%, and fungicides –84.5% [1]. The absence of imported biological preparations is explained by their limited shelf life, problems during transportation, and higher prices, compared to those of domestic preparations. Therefore, industrial production and use of domestic microbiological means of plant protection on the territory of Russia at the end of the 20th century and at the beginning of the 21st century are gradually starting to increase [2, 3]. This is also due to increasing interest of consumers of crop breeding to the issues of environmentalization and biologization of agriculture.

With all the variety of crops represented in the State Collection of Microorganisms, the number of registered preparations based on them and presented in the "State Catalog of Pesticides and Agrochemicals" in 2014-2016 includes only 15-18 genera.

Among biofertilizers, cultures of microorganisms are represented by the same types of bacteria as among pesticides, including *Bacillus subtilis* fungi of genus *Trichoderma* (product Geostim, LTD Biotekhagro), bacteria of genus *Agrobacterium* (preparation Risoagrin, LLC Biofabrika), bacteria *Bradyrhizobium japonicum* (preparation Nitragin, LLC Invivo), *Azotobacter chroococcum* (preparation Azotovit, JSC Industrial innovations), *(Bacillus mucilaginosus* (preparation Fosfatovit, JSC Industrial innovations), etc.

Most efficient in crop production are biological products based on bacteria *Azotobacter chroococcum* [4-6].

The use of preparations based on *A. chroococcum* for seed treatment, and as nitrogenous fertilizers, has been known since the 30s of the last century. These bacteria synthesize and produce vitamins and biologically active substances consumed by plants. These include biotin, heteroauxin, gibberellin, nicotinic and pantothenic acids [7-10]. In addition, its antagonistic activity has been discovered against pathogens of plant diseases that occur in the seeds and planting material, as well as in the soil (fungi of genera *Fusarium, Alternaria, Penicillium, Helminthosporium*) due to the ability to emit fungicidal substances of the anisomycin group [11, 12]. Bacteria *A. chroococcum* are demanding to nutrition. They need sources of phosphorus in the soil. At the same time, in soils that are rich in humus, and with limited amount of plant residues, their reproduction is hampered. They are used as indicators for checking the presence of phosphorus and potassium in the soil. They need sulphur, magnesium, and calcium. *A. chroococcum* actively consumes substances that are formed during cellulose decomposition, assimilates molecular nitrogen in aerobic conditions, providing available forms of nitrogen to plants. Nitrogen fixation is a reduction process performed due to the work of the nitrogenase complex of Azotobacter. This requires the presence of molybdenum and vanadium in the nutrient fluid, since these elements are included into the coenzyme systems of nitrogen fixation enzymes. These bacteria develop well on nitrogen-free Ashby, Beyerinck with the introduction of trace elements of boron, molybdenum, etc., forming mucoid colonies. In adverse conditions (exposure to an environment with an insufficient number of factors for their nutrition) station of their polymorphic properties occurs, and the microorganisms themselves lose the ability to nitrogen fixation [13, 14]. Polymorphism of *Azotobacter* is manifested in various shapes of cells, from baculiform to coccal. In the course of aging, cells of *A. chroococcum* can synthesize brown pigment *(chroo).* The pH level acceptable for the life of *A. chroococcum* is between 5.5 and 8.2, but the optimum level is 7.2. In acidic medium, it develops with difficulty, and with the pH level below 4.5 and above 9.0 the growth of *A. chroococcum* virtually stops. The optimum temperature for development of these bacteria is 25-30^{o}C. However, at lower temperatures they also preserve their viability. The number of these microorganisms does not decrease even in northern regions during winter [7, 13]. However, in the area of regular and southern black soils without irrigation, the ability of Azotobacter to actively proliferate in the soil is quite limited [11]. In this respect, development of the methods of using this valuable organism in crop breeding is quire relevant.

Previously, the authors have developed a scheme of low-tonnage production of microbiological preparations at farms in the vicinity to the location of consumption [15].

Further work was aimed at improving the method of obtaining the microbiological preparation based on strain *A. chroococcum A. chroococcum VKM B-1616.*

The main task was determining the influence of various amino acids (methionine, glycine, lysine, phenylalanine) on increasing the final titer of the preparation of *A. chroococcum VKM B-1616.*

MATERIAL AND METHODS

The objects of the research were bacteria *A. chroococcum VKM B-1616* (from the collection of FSBEI HE Kuban State Agricultural University), and bacteria of the same strain lyophilically dried at the production base of LLC Biom PRO, with the titer not less than $1x10^7$ CFU/g.

The experiments were performed at the laboratories of the Department of Physiology of Plant Biochemistry and Climatic Chambers with Artificial climate of the FSBEI HE Kuban State Agricultural University in 2011–2015.

The quantitative accounting and comparative assessment of the culture fluids of microorganisms were made using the method of Koch dilutions by planting appropriate dilutions of cultural fluids on the nutrient media regulated by the normative documents. Preparation of initial suspensions and a series of 10-fold dilutions were made according to GOST ISO 7218-2015 [16] and GOST 26669. The number of microorganisms CFU/ml (g) was calculated according to the formula shown in GOST ISO 7218-2011(2016):

$$N = \frac{\sum C}{V \bullet 1{,}1d}, \quad (1)$$

where $\sum C$ was the sum of colonies on two plates from two successive dilutions in which at least one Cup contained less than 10 CFU;

V was the volume of seed material introduced into each cup, cm^3;

D was the dilution coefficient.

The nitrogen-fixing activity was determined by the method of Kjeldahl according to GOST R 53951-2010.

The duration of lag phase was determined on the moment of appearance of first growth points in the future colonies by microbiological planting of cultural fluids obtained from bran environments, onto agar media (No. 1, GRM-agar, Ashby). The end of the phase of logarithmic growth was determined at the time when the number of CFU/ml of the culture liquids seeded onto agar media virtually stoped growing. The stationary phase was determined by the absence of growth in the number of CFU/ml, which, starting after 96 hours of cultivation, remained almost constant when cultural liquids were planted onto agar media for bacteria.

The quantitative parameters that characterized growth of the culture were determined by the following formulas [17]:

- the number of divisions of cells during the logarithmic phase - n

$$n = \frac{\lg N_t - \lg N_0}{\lg 2} \quad (1)$$

where N_0 was the initial number of cells, N_1 was the number of cells at the end of the logarithmic phase;

- the division rate constant - ν

$$\nu = \frac{n}{t} \quad (2)$$

where *n* was the number of cell divisions per period of the logarithmic phase,

t was the duration of the logarithmic phase,

- the generation time - g

$$g = \frac{t}{n} = \frac{1}{\nu} \quad (3)$$

In case of classical scheme of microorganisms' cultivation, where all components of the nutritional medium were simultaneously introduced into the nutrient medium along with the culture of microorganisms, the exponential (logarithmic) phase of bacteria cultures' growth lasted only 24 hours.

The following variants of culture media have been studied:

1. Classic nutrient medium (Beyerinck medium). In preparing classical culture media, General instructions for preparation and sterilization of culture media according to GOST ISO 7218-2011 and GOST ISO 11133-2-2011 were used.

2. Bran and sucrose (granulated sugar) medium.

3. Bran and sucrose (granulated sugar) media with the addition of various salts for the work of the enzymatic complex and growth activators. Growth activators introduced into the culture media for studying their effect on the microorganisms were amino acids: methionine, glycine, lysine, and phenylalanine. The compositions of the media are shown in Tables 1 and 2.

The total duration of the culture cultivation period was 120 hours.

RESULTS

The results of the experiments (Table 1) show that the nutrient medium of the developed composition – sucrose and wheat bran in the ratio of 1.25:2.0 - prepared according to the 2-phase fractional scheme [15] is sufficient for ensuring growth properties of *A. chroococcum.* By the end of the cultivation, the titer of microorganisms on this medium was $1.9x10^8$ CFU/ml, i.e. it was the same as on the Beyerinck medium - $2.0x10^8$ CFU/ml (Table 1).

Introduction of sodium and ammonium molybdates into the sucrose and bran medium each at the rate of 0.005 g/1,000 ml of medium yields similar results. However, in this case, the use of ammonium and sodium molybdate is not considered as growth activator, and is only used as a component for the nitrogen-fixing activity of the bacteria enzyme complex. The nitrogen-fixing activity by Kjeldahl was 13.45 mg of nitrogen per 1 g of the used sugars when added to the culture medium of trace elements by Fedorov, and 12.87 and 13.25 mg of nitrogen per 1 g of the used sugars when sodium molybdate and ammonium molybdate were added to the medium, respectively.

Table 1 – The number of CFU and the nitrogen-fixing ability of *A. chroococcum VKM B-1616* with growing on the nutrient media of various compositions (growth over 5 days)

Variants of the experiment	pH of the nutrition medium		CFU/ml	Nitrogen-fixing activity, mg of nitrogen/1 g of sugar
	start	end		
1. Beyerinck medium	7.0	5.89	$2.0x10^8$	15.34
2. Medium: sucrose + bran (12.5:20.0/1,000 ml)	7.0	5.70	$1.9x10^8$	12.15
3. Medium: sucrose + bran (12.5:20.0) + trace elements according to Fedorov (1 ml/1,000 ml)	7.0	5.73	$2.0x10^9$	13.45
4. Medium: sucrose + bran (12.5:20.0) + Na_2MoO_4 (0.005 g/1,000 ml)	7.0	5.79	$2.0x10^9$	12.87
5 Medium: sucrose + bran (12.5:20.0) + $(NH_4)_2MoO_4$ (0.005 g/1,000 ml)	7.0	5.77	$2.0x10^9$	13.25
LSD_{05}			**$6.7x10^7$**	**0.99**

Table 2 – Changes in the quality of the *A. chroococcum biological product VKM B-1616* under the influence of introducing various growth activators of microorganisms into the nutrient medium (growth over 5 days)

Variants of the experiment	pH of the nutrition medium		CFU/ml
	start	end	Average value*
6 Medium: sucrose + bran (12.5:20.0) + methionine (0.1 g/1,000 ml)	6.98	5.70	$1.6x10^9$
7 Medium: sucrose + bran (12.5:20.0) + glycine (0.1 g/1,000 ml)	7.0	5.80	$4.8x10^8$
6 Medium: sucrose + bran (12.5:20.0) + lysine (0.1 g/1,000 ml)	7.0	5.8	$1.6x10^8$
6 Medium: sucrose + bran (12.5:20.0) + phenylalanine (0.1 g/1,000 ml)	7.0	5.85	$6.5x10^7$
10 Medium: sucrose + bran (12.5:20.0) + K_2HPO_4 (0.005 g/1,000 ml)	7.0	5.83	$2.0x10^8$
11 Medium: sucrose + bran (12.5:20.0) + K_2HPO_4 (0.005 g/1,000 ml)	7.0	5.80	$1.0x10^9$
12 Medium: sucrose + bran (12.5:20.0) + K_2HPO_4 (0.15 g/1,000 ml)	7.0	5.70	$1.5x10^9$
13 Medium: sucrose + bran (12.5:20.0) + K_2HPO_4 (0.2 g/1,000 ml)	7.0	5.73	$1.5x10^9$
14 Medium: sucrose + bran (12.5:20.0) + K_2HPO_4 + KH_2PO_4 (0.05 g/1,000 ml)	7.0	5.77	$1.0x10^9$
15. Medium: sucrose + bran (12.5:20.0) +methionine (0.1 g/1,000 ml)+ $(NH_4)_2MoO_4$ (0.005 g/1,000 ml)	7.0	5.80	$2.0x10^9$
Medium: sucrose + bran (12.5:20.0) +methionine (0.1 g/1,000 ml)+ Na_2MoO_4 (0.005 g/1,000 ml)	7.0	5.70	$2.0x10^9$
LSD_{05}			**$4.5x10^7$**

*Intervals correspond to the upper and lower limits of the confidence interval at the level of 95% (0.95) according to GOST 10444.12-2013, Appendix B.

Production tests showed that when dry (freeze-dried) formulations of microorganisms were used, bacteria passed their life cycle in the following time: lag phase - 16...24 hours, the phase of logarithmic growth + 20...24 hours, growth deceleration phase + 6...8 hours, the stationary phase that lasted about 48 hours, and the dying place occurred after 100 to 120 hours of growth.

When calculating the growth of microorganisms in obtaining preparations in industrial conditions in accordance with the periodic 2-phase scheme of cultivation [15], the following should be noted. At the initial moment, 1 kg of dry starting inoculum with the titer of not less than $1x10^7$ CFU/g is introduced into 1,000 l of the 1.25% sucrose solution. Accordingly, the initial titer of the culture decreases in this volume of the medium to $1x10^4$ CFU/ml. In this case, the lag phase takes 24 hours, since the cells are in stressed (dried) state. This period is characterized by almost constant number of cells (does not reduce in working with these cultures) and is $1x10^4$ CFU/ml during almost the entire period. In addition, the optimal culture to environment ratio is between 1:10 and 1:100 [7], and in this case, dilution of culture is determined by the ratio of 1:1,000.

Thus, in case of single-phase scheme of introducing any components of the nutrient medium, the number of *A. chroococcum VKM B-1616* cell divisions (*n*) is the following: (7-4)/0.3=10.

The constant of division rate *V* over the 24 hours of the logarithmic phase for *A. chroococcum VKM B-1616* is 0.42.

In cultivation of *A. chroococcum VKM B-1616* according to the 2-phase scheme with the introduction of bran after 24 hours of the target culture growth, the same indicators will be: *n*=15.93, and *V*=0.66.

At this stage of the logarithmic phase, after the introduction of bran, diauxie (additional temporary growth phase of the culture) occurs, after which adaptation of the culture to wheat bran starts, since a new source of nutrition appears.

Taking into account the moments of 24 and 48 hours in taking the logarithm of the CFU/ml number, the generation time according to the first scheme is 7.1 hours, and according to the second - 3.6 hours.

Into other samples of the media for growing A. *chroococcum*, amino acids were added: methionine, glycine, lysine, phenylalanine at the dosage of 0.1 g/1,000 ml of the medium (variants 6-9). The choice of these amino acids was determined by the need to study their suppressing or growth promoting action on the Azotobacter genus. It is known that bacteria of the Azotobacter genus do not digest monocarboxylic amino acids, namely, lysine, arginine, histidine, alanine and glycine [7]. Methionine, glycine, lysine and phenylalanine amino acids are used by agricultural companies for seed treatment along with the liquid microbiological preparation based on *A. chroococcum* [18]. Therefore, it was important to determine their influence on the *A. chroococcum bacteria.*

The need of *A. chroococcum* in phosphorus-containing organic or inorganic compounds for its reproduction is known. In the Ashby and Beyerinck media, the amount of $K_2HPO_4^-$ is 0.2 g/1,000 ml. Phosphorus content in the bran is 1,013 mg/100 g. After the introduction of 20 g of bran, the amount of phosphorus in the nutrient medium will be 0.2026 g/1000 g, which is sufficient and corresponds to the content of phosphorus compounds in the Beyerinck medium. However, to determine the possibility of increasing the number of CFU of *A chroococcum VKM B-1616* , K_2HPO_4 was additionally introduced into the medium in the quantity of 0.050; 0.100; 0.150; 0.200 g/1,000 ml of medium (variants 10-13), as well as $K_2HPO_4^+ KN_2OO_4$ in the amount of 0.050 g of each per 1,000 ml of the medium (variant 14). The results are shown in Table 2.

The data show that adding glycine into the medium increases the yield of microorganisms 2.5 times, and adding lysine does not have significant effect. Phenylalanine reduces the yield of microorganisms 3 times. The maximum positive effect was observed in the presence of the aliphatic sulfur-containing amino acid, methionine: the number of viable cells increased 8 times - from $1.9x10^8$ CFU/ml in the sucrose and bran medium and $2.0x10^8$ CFU/ml in the Beyerinck medium to $1.6x10^9$ in the bran medium with the addition of methionine. With that, neither reduction of the lag phase duration, nor elongation of the stationary growth phase has been detected.

In case of using a nutrient medium with addition of methionine and salts of molybdenum, the number of *A. chroococcum VKM B-1616 (n) cell divisions* and the speed constant (V) in the logarithmic phase, calculated according to formulas (1) and (2) was equal to: *n*=(9.2-4)/0.3=17.33 and *V*=17.33/24=0.72 (Figure 1).

And the "generation time" in the phase of exponential growth *(q)* calculated by formula (3) was 2.4, in contrast to the similar indicator in case of 1-phase scheme equal to 7.1, and 2-phase scheme - equal to 3.6 (Figure 1).

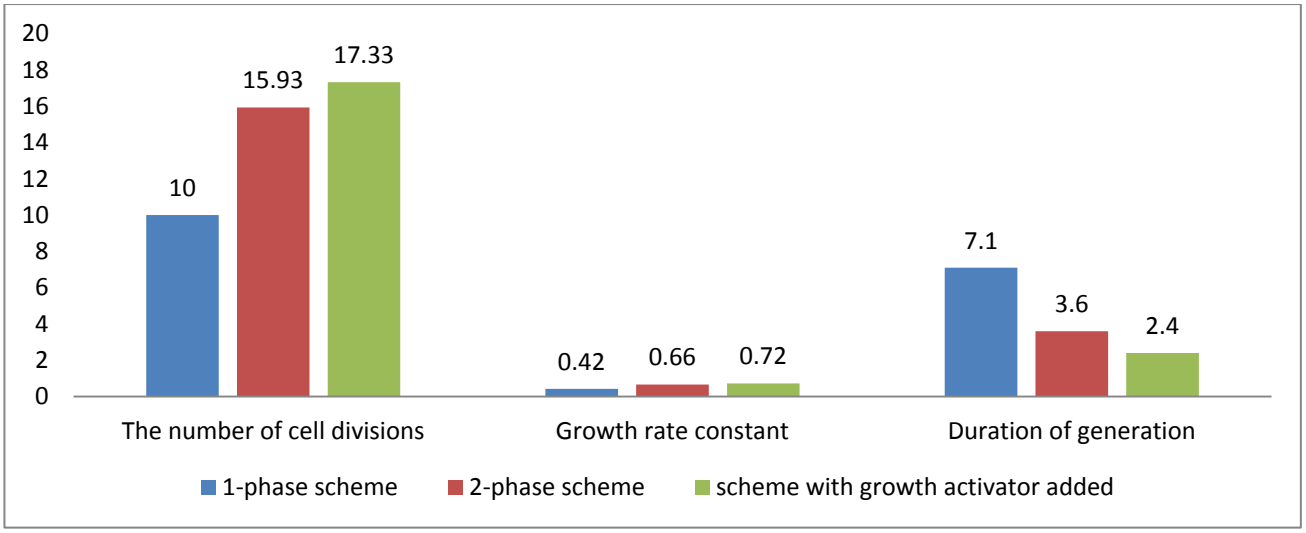

Figure 1– Kinetic parameters of *A. chroococcum VKM B-1616* logarithmic growth phase with the classic 2-phase scheme and the 2-phase scheme with the introduction of growth activator

Discussion

Analysis of the nutritional value of bran showed that medium based on it was able to fully satisfy the needs of *A. chroococcum* in nutrients [15]. Additional introduction of disubstituted, as well as monosubstituted phosphated potassium to the nutrient medium in the dosage of 0.150 g/1,000 ml of the medium, compared to the reference sample (Beyerinck medium), increases the number of cells to $1.5x10^9$ CFU/ml. Further increase to 0.200 g/1000 ml of the medium does not increase the number of the *A. chroococcum VKM B-1616* cells. However, the overall content of phosphorus in wheat bran of 1,013 mg/100 g of bran is enough and additional introduction of phosphorus compounds into the culture medium is not required.

Of the studied amino acids, methionine had the maximum effect on the growth of the *A. chroococcum VKM B-1616* culture. The literature has evidence that methionine can influence physiological processes in plants. Therefore, it has been shown that exogenous methionine accelerates the growth of seedlings [19], and reduces the toxic effect of herbicide 2,4-D on wheat [20]. However, no data about the growth promoting action of sulfur-containing compounds on the bacteria of genus Azotobacter have been found.

The experiments do not allow drawing conclusions about the mechanisms of methionine stimulatory effect. Probably, this amino acid is engaged in the plastic exchange, and promotes growth of the biomass, or it is a regulator of growth processes. The latter assumption is supported by significant reduction of the generation time - from 7.1 to 2.4 hours.

Conclusions

Thus, according to the data of the research, the optimum medium for cultivating strain *A. chroococcum VKM B-1616* is the medium of the following composition (g/1,000 ml): sucrose – 12.5; wheat bran – 20/0; methionine – 0.1; Na_2MoO_4 $(NH_4)_2MoO_4$ – 0.005.

It should be noted that the great advantage of the proposed technology of 2-phase preparation of microbiological products is the ability to quickly and efficiently respond to a specific complex of phytopathogenic factors developing at a farm.

Production tests performed at LLC SKIF (village Starominskaya, Krasnodar region), LLC Dzhumaylovskoye (village Kalininskaya, Krasnodar Krai), LLC AE Temizhbekskoye (Stavropol Krai), LLC Aksai land (Rostov region) and LLC Agronova (Volgograd region) showed high efficiency and reliability of the developed technological schemes.

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