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Conditions for the Cultivation of New *Bacillus* Bacteria being Micro Bioproduct Producers

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Optimal conditions for cultivation of *B. subtilis* BZR 336g and *B. subtilis* BZR 517 strains have been found in the course of the study: temperature, pH, carbon and nitrogen nutrition sources. As a result of the research some original samples of optimized culture media were obtained to produce liquid agent cultures (LAC) with an optimal number of colony-forming units combined with a high anti-fungal activity.

Key words: Bacteria-antagonists, *Bacillus subtilis*, Cultivation conditions, Temperature, pH, sources of nutrition.

Most cultivars of agricultural crops in the average implement only 20-25 % of the genetic potential of productivity. With protection from diseases, pests and weeds they can produce a much greater yield. The average world level of losses due to infection of agricultural plants with phytopathogenic microorganisms is estimated at 12 %[1]. To protect plants from diseases chemical fungicides are widely used. Their application efficiency can be as high as 100%, but there arise a number of problems, the main of them being environmental pollution and toxicity of the products[2]. In this regard, it is most important to use environmentally safe methods of plant protection against pathogenes. One of the ways of solving this problems of plant protection is the shift towards creation and application of microbial agents.

Among aerobic spore-forming bacteria it is *Bacillus* genus that is most widely used as the basic substance for biologics against plant diseases[3, 4]. Bacteria of *Bacillus subtilis* species are highly productive and promising representatives of *Bacillus* genus in microbiological industry[5-7].

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Bacterial fungicides based on *Bacillus* genus strains possess a number of positive features: a high antifungal activity[8-10]; a rapid antagonistic effect[11-12]; the bacilli's ability of colonizing various parts of the plant and formation of biofilms in the rhizosphere and on the leaf surface[13]; the absence of pathogen resistance to bacilli and compounds formed by them[14]; the possibility of their use at different stages of plant development, as well as (including) for seed and soil treatment; their compatibility with chemicals[15].

Nowadays there are many *Bacillus* genus bacteria-based biologics used worldwide: Avogreen (*B. subtilis*), Ballad (*B. pumilus*), Bio safe (*B. subtilis*), Biosubtilin (*B. subtilis*), Botrybel (*B. velezensis*), Cease (*B. subtilis*), Companion (*B. subtilis*), Kodiak (*B. subtilis*), Ecoshot (*B. subtilis*), EcoGuard TM (*B. licheniformis*), FZB24WG (*B. subtilis*), Rhizo Plus (*B. subtilis*), HiStick (*B. subtilis*), RhizoVital42 (*B. amyloliquefaciens*), Subtilex (*B. subtilis*), Pro-Mix (*B. subtilis*), Rhapsody (*B. subtilis*), Serenade (*B. subtilis*), Sonata (*B. pumilus*), Sublic (*Bacillus sp.*), Yield Shield (*B. pumilus*) (the USA, Canada, China, India, etc.)[16].

Despite the above advantages and numerous research works in the field, the quantity of ready-made biopreparations in the Russian

market is quite insufficient. In 2013 the following *Bacillus*-based biopreparations were allowed to be used at the Russian Federation territory: Bactofit (*B. subtilis*), Phytosporin-M (*B. subtilis*), Alirin B (*B. subtilis*), Gamair (*B. subtilis*), Vitaplan (*B. subtilis*), Bisolbisan (*B. subtilis*)[17]. Such a poor choice is, among other things, due to the absence of up-to-date standards and biotechnologies for obtaining biopesticides[18].

It should be born in mind that the active element of biologics are living cells of microorganisms. That is why they have a number of special characteristics:

- titer and biological activity decrease in the process of long storage[19];
- sensitivity to environmental factors[20];
- loss of activity by biologics producer strains[21];
- critical or limited time of application[22, 23];
- short residual time of activity[5];
- pollution by foreign micro biota[24].

At the biologics production stage the bioagent's sensitivity to environmental factors (components of nutrient media) is of utmost significance. Thus, temperature influences the velocity of cell reactions, the nature of metabolism, nutrition demands and the biomass composition. The culture pH level influences the end-products of anaerobic transformation of carbon and energy sources, affects the medium composition and the nature of surface of the microorganisms, tells on acids and bases dissociation. The organic matter carbon is used by bacteria for construction of their cells and as a source of energy for cell material biosynthesis, for the cell's growth and motility. The nitrogen source metabolism ensures mainly synthesis of proteins, nucleic acids and cell membrane polymers[25-27].

Therefore, it is a very important (actual) task to ensure the vitality, biological activity and purity of the producing strains cell culture, to develop and improve the production technologies of biopreparations based on them, as well as to improve production technologies taking into account trophic demands of microorganisms, and their resistance to all kinds of effects. With optimal combination of all components in qualitative and quantitative relation the medium provides sufficiently quick growth and development of microorganism population and is considered to be well-balanced[28, 29].

Thus, one of the essential branches of biotechnology today is the search of optimal conditions for cultivating producer strains of biologics: choosing new substrates and nutrition sources, improvement of cultivation methods, studying vitality and activity of microorganisms under different cultivation conditions.

Creating effective biological plant protection involves the development of laboratory regulations of mass production of biologics based on the study of physiological features of promising strains-producer. Therefore, the purpose of the present work was to study the quantitative patterns of promising bacterial strains population growth in batch cultivation conditions, and evaluation of their antifungal activity, which subsequently will solve the problem of optimization of processing methods of use of biologics.

EXPERIMENTAL

Bacillus subtilis strains

The object under study was new producer strains of biopreparations test species for protecting winter wheat against Fusarium root rot pathogen from the ARRIBPP microorganism operation collection: B. subtilis BZR 336 g, B. subtilis BZR 517[30-34], the phytopathogenic fungus *Fusarium graminearum* Schwabe test-culture.

Determination of optimal conditions of cultivation

The incubation was carried out in thermostatized systems of cell cultivation (180 rot./min) New Brunswick Scientific Excella E25 (USA) for 48 hours. The batch cultivation was performed in conic flasks (350 ml) with the culture medium volume of 100 ml and pretreatment with the sown (stock) culture (2.0 % of the culture medium volume). The stock culture was obtained by means of introducing agar blocks with the strains under study into the conic flasks and their successive cultivation[35].

To determine the optimal temperature of cultivation the strains were grown at temperatures 20, 25, 30 and 35°C. To determine the optimal culture medium pH the strains were grown on liquid potato glucose substrate (PGS) (500.0 ml potato broth, 20.0 g glucose per litre, Russia)[36]. By adding lactic acid or alkaline (4 N solution of NaOH, Russia) the substrate reaction was kept within the 3.0, 6.0, 8.0 and 10.0 pH measured by a pH meter Sartorius PB-11 (Germany).

Determination of the optimal nutrition sources

As the carbon sources sucrose, glucose, molasses and glycerol were added in the test media. In the study of carbon sources nitrogen sodium nitrate served as the unchanged nutrition component. In determining the optimal sources of nitrogen nutrition peptone, $NaNO_3$, yeast and corn extracts were tested with glucose as the only (constant) carbon source. Chapek medium for bacteria was used as basic (0.5 g KCl , 0.5 g $MgSO_4$, 1.0 g K_2HPO_4, 3.0 g $CaCO_3$, 1.0 ml of 1% solution $FeSO_4$, 2.0 g $NaNO_3$, 20.0 g glucose per litre, all the components of the Russian manufacture)[37]. The concentrations of the introduced carbon and nitrogen sources, as well as the composition of the (original) culture medium (orig.), as elements of the production know-how for new biopreparations, are the objects protected as a trade secret All-Russian Research Institute of Biological Plant Protection (order number 42-p of 28 November 2012). As a control, the King's B liquid medium (KB) (20.0 g peptone, 15.0 g glycerol, 1.5 g $MgSO_4$, 1.5 g K2HPO_4 per litre, all the components of the Russian manufacture) was used[38] and the PGS.

Determination of the number of colony forming units

Upon completion of the cultivation in all the experiments the number of bacterial cells was determined. To study the quantitative patterns of population growth of the strains under different conditions the Koch method was used[39]. The determination of number of cells by this method consists of three stages: preparation of dilutions, sowing on the nutrition medium in PE. Calculation of the grown colonies was performed using a system for automatic counting of colonies Color Qcount, Spiral Biotech (USA).

Determination of the antagonistic activity

Determination of the antagonistic activity of the strains under study was performed by double (counter) cultures method[40, 41] on potato-glucose agar (PGA), agarized KB medium and the optimized medium (orig.). An agar block with the mycelium of a pathogen was inoculated into a Petri dish, with the bacterial strain deposited by the stroke method at the distance of 6 cm from the pathogen block. The cultures were incubated for 20 days at + 28 °C. Pure cultures of bacteria and fungus pathogen sown separately were used as control options. The recordings were taken at the 5-th, 10-th, 15-th and 20-th days. The type of relationship of the fungus and bacteria was marked: the presence or absence of zones, their size, changes in color, density, thickness and direction of the pathogen mycelium growth. The degree of inhibition of the pathogen mycelium growth was determined according to the formula[42]:

$$I = (1 - (A / B)) \times 100,$$

with I being the inhibition of the pathogen mycelium growth, %;

A – fungus growth in the option, mm;

B – fungus growth in the control, mm.

RESULTS AND DISCUSSION

Temperature is an important factor for the growth of microorganisms. The investigation showed that a high density (concentration) of cells in the replication with the strain *B. subtilis* BZR

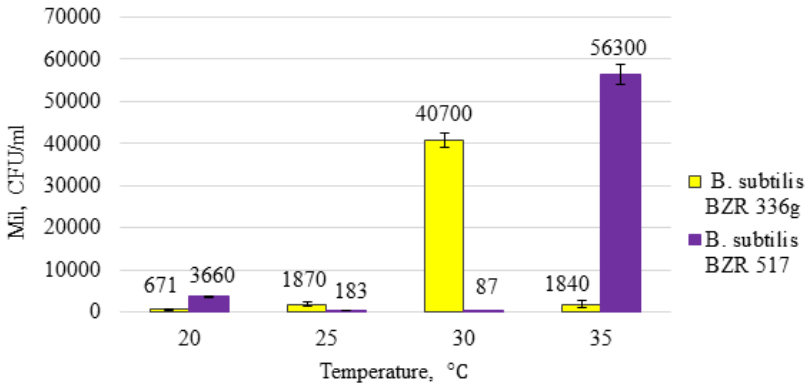


Fig. 1. Temperature effect on the growth of strains *B. subtilis* BZR 336g and *B. subtilis* BZR 517 in the process of batch cultivating

336g was marked at the cultivation temperature of 30 °C making 4.1 x 10^{10} CFU/ml. The highest LA titer of strain *B. subtilis* BZR 517 was marked at the temperature of 35 °C: 5.1 x 10^{10} CFU/ml. Based on the data obtained it is safe to conclude that the most optimal temperatures for bacillary strains are these: 30.0 and 35.0 °C (Fig. 1).

The pH is of critical significance for cultivation of microorganisms. When it changes to the worse, the microorganisms cease to grow, even with all other conditions being optimal.

The optimal pH for growth of the strain *B. subtilis* BZR 336g is established to be 6.0 and 8.0: 1.6 x 10^{8} CFU/ml and 1.7 x 10^{8} CFU/ml, correspondingly. The pH values of 3.0 and 10.0 proved to be limiting. The highest titer for strains *B. subtilis* BZR 517 was marked at pH 10.0: 1.0 × 10^{9} CFU/ml. The number of colony-forming units in LA based on this strain was growing proportionally to the increase of the medium pH level (Fig. 2).

The maximum cell concentration in prototype LA based on the strain *B. subtilis* BZR 336g was observed on the medium with molasses as the carbon source: 1.1 x 10^{9} CFU / ml. In options with the addition of glucose, sucrose and glycerol the LA titer was lower by two orders . For strain *B. subtilis* BZR 517 medium supplemented with molasses was also optimal for growth, the LA titer was 3.5 x 10^{11} CFU / ml (Fig. 3) . These results are explained by the fact that molasses being a byproduct of sugar production contains not only sucrose, but other organic nitrogen-free substances and minerals.

High LA titer in the option of the strain *B. subtilis* BZR 336g was observed on the nutrient medium with peptone, yeast extract and corn used as the nitrogen source: 1.5-5.7 x 10^{8} CFU/ml. These components were also optimal for strain *B. subtilis* BZR 517 – 2.8-5.5 x 10^{8} CFU/ml (Fig. 4). In all

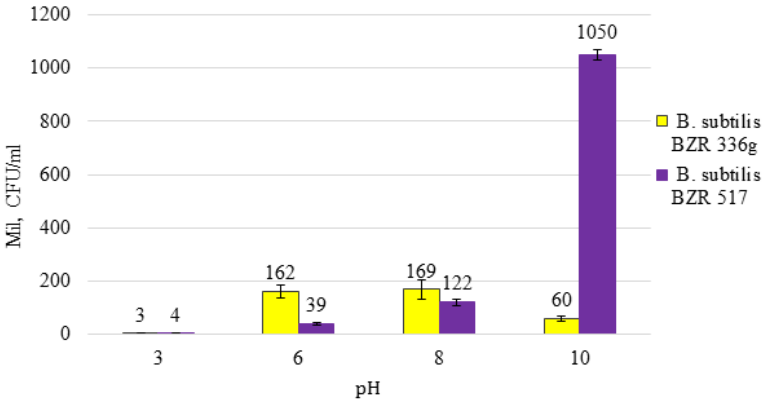


Fig. 2. pH effect on the growth of strains *B. subtilis* BZR 336g and *B. subtilis* BZR 517 in the process of batch cultivating

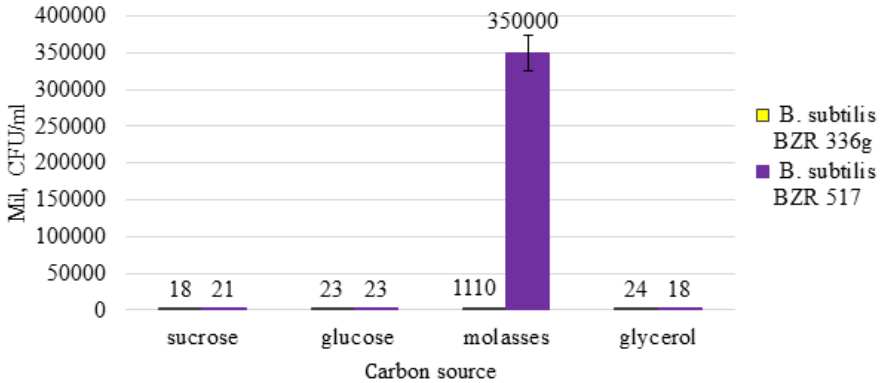


Fig. 3. The effect of carbon nutrition sources on the growth of strains *B. subtilis* BZR 336g and *B. subtilis* BZR 517 in batch cultivation process

likelihood, this is due to the fact that peptone is a product obtained by the action of proteolytic enzymes, making it more available for digestion, while in the composition of the yeast extract and corn there are vitamins, organic acids, macro -and micronutrients[39].

Based on the data obtained, we selected the first samples of the original optimized culture media. As a control the KB culture medium was used, on which the biological agents under study produced from natural sources were actively growing and exhibited a high antifungal activity. The studies found that the number of colony-forming units of LA based on the strain *B. subtilis* BZR 336g on the optimized medium was significantly higher than in the KB and PGS media and made 8.7 x 10^{10} CFU/ml. For strain *B., subtilis* BZR 517 the optimized nutrient medium was also preferable (effective) by this criterion compared to standard media: the LA titer was 7.2 x 10^{10} CFU/ml (Table 1).

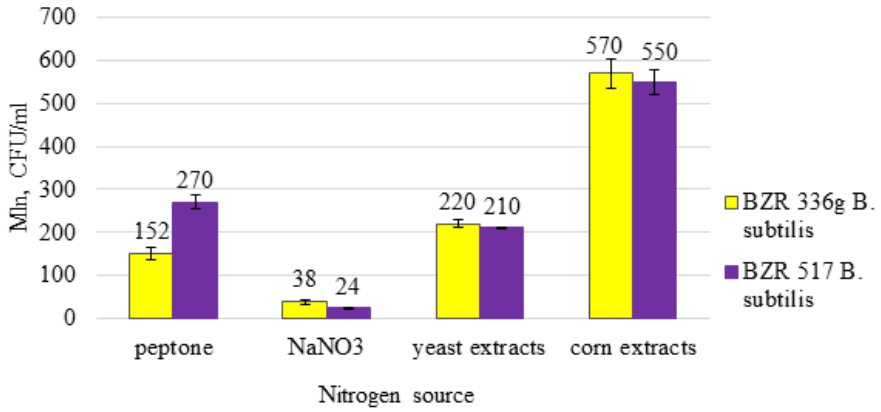


Fig. 4. The effect of nitrogen nutrition sources on the growth of strains *B. subtilis* BZR 336g and *B. subtilis* BZR 517 in batch cultivation process

Table 1. Growth of strains *B. subtilis* BZR 336g and *B. subtilis* BZR 517 on different nutrient media in batch cultivation process

Strain	Nutrient medium, LA titer, CFU/ml		
	KB	PGS	Optimized medium (orig.)
B. subtilis BZR 336g	(5,7±0,06) × 10^7	(1,3±0,14) × 10^8	(8,7±0,66) × 10^{10}
B. subtilis BZR 517	(3,7±0,14) × 10^8	(1,8±0,07) × 10^8	(7,2±0,42) × 10^{10}

* - the table shows the average values and standard deviation

It is well-known that the development of biotechnologies to obtain complex action biofungicides demands LA with optimum density of microbial cells in combination with high concentrations of antifungal agents. Therefore, at the next stage we studied antifungal activity of producing strains of biological products on various nutrient media.

For strain *B. subtilis* BZR 336g maximum antifungal activity was noted on the optimized medium, and made 90.0% on the fifth day, 83.6 % on the tenth day and 78.2% on the fifteenth day. On PGS and KB medium the degree of inhibition of the pathogen was increased to the tenth day of incubation, but remained significantly lower than in the option with the optimized medium. By the fifteenth day the antifungal activity in these media began to decline (Fig. 5). It is important to note for that matter, that only on the optimized medium, this strain has a high mobility: as early as on the fifth day of co-incubation the biological agent occupied the full area of the nutrient medium blocking the pathogen growth (Fig. 7).

The degree of inhibition of *F. graminearum* in the option with strain *B. subtilis* BZR 517 increased by the tenth day in all nutrient media, but no significant difference in antifungal activity was observed, as is in the case with strain

of *B. subtilis* BZR 336g (Fig. 6). However, as in the case of the strain *B. subtilis* BZR 336g, *B. subtilis* BZR 517 on the optimized medium had a higher mobility (Fig. 7).

Furthermore, among the impact characteristics of metabolites of the active bacteria strains on *F. graminearum* the following should be noted: in the antagonistic action area of bacteria in some options the lysis of an already formed mycelium growth was observed, with inhibition and change of colour of the pathogen mycelium.

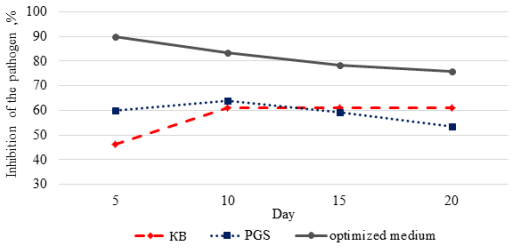


Fig. 5. Antifungal activity of strain *B. subtilis* BZR 336g against *F. graminearum*

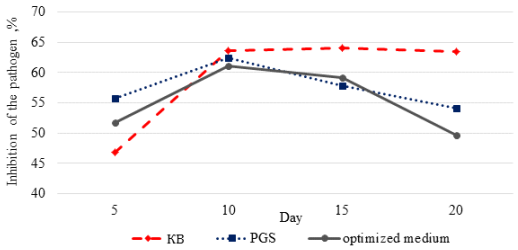
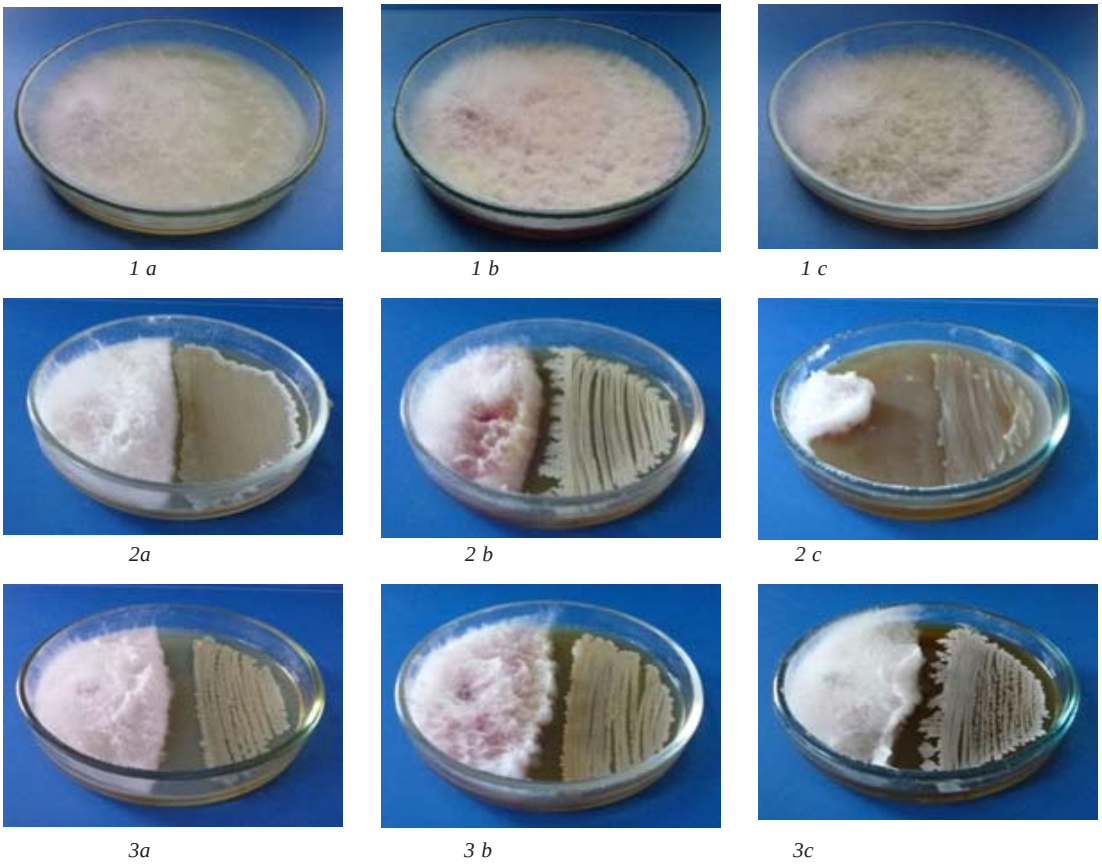


Fig. 6. Antifungal activity of strain *B. subtilis* BZR 517 against *F. graminearum*



a - KB; *b* - PGS ; *c* - optimized medium (orig.); 1 - control (pure culture *F. graminearum* without the antagonist); 2 - double culture *F. graminearum* and *B. subtilis* BZR 336g; 3 - double culture *F. graminearum B. subtilis* BZR 517.

Fig. 7. Antifungal activity of strains *B. subtilis* BZR 336g and *B. subtilis* BZR 517 against *F. graminearum* on different nutrient media

Thus, as a result of the research, the optimal conditions for cultivation of strains *B. subtilis* BZR 336g and *B. subtilis* BZR 517 were found, original optimized culture media samples were obtained, providing the LA with the required number of colony forming units (no less than 1.0 x 10^{10} CFU/ml) in combination with a high antifungal activity and mobility.

ACKNOWLEDGEMENTS

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REFERENCES

1. Minina, T.S., Zakharova, R.Sh., Urazbakhtina, N.A., Khairullin, R.M. New endophytic *Bacillus subtilis* strains as base for biofungicides. *Kazan SAU Newslett.*, 2009; **2**(12): 118-123.
2. Pitan, B., Schubert S. Decline in leaf growth under salt stress is due to an inhibition of H+ pumping activity and increase in apoplastic pH of maize leaves. *J. Plant Nutr. Soil Sci*., 2009; **172**: 535-543
3. Shternshis, M.V. Trend of development of microbial plant protection means biotechnology in Russia. *Tomsk SU Bull. Biol.*, 2012; **2**(18): 92-100.
4. Barriuso, J., Solano, B.R., Lucas, J.A., Lobo, A.P., Villaraco, A.G., Mañero, F.J. Ecology, Genetic Diversity and Screening Strategies of Plant Growth Promoting Rhizobacteria (PGPR). *J. Plant Nutr*., 2008; **3**: 1-17.
5. Pashkevich, Ye. B. Biological grounds for creation and usage details of biologics containing *Bacillus subtilis* for plant protection against phyto-pathogens. *Agrochem. Ecol. Probl*., 2009; **2**: 41-47.
6. Asaturova, A.M., Dubyaga, V.M., Tomashevich, N.S., Zharnikova, M.D. Selection of promising biologic control agents for winter wheat protection against Fusarium pathogens. *KubSAU Electron. Multi-discipl. Sci. J*. 2012. 75(01).
7. Asaturova, A.M., Dubyaga, V.M., Tomashevich, N.S., Zharnikova, M.D., Zhevnova, N.A., HomyAk, A.I. Development of environmentally friendly bacterial biopreparations for the protection of fall wheat from *Fusarium* pathogens and other diseases. In *10th International conference on plant diseases 2012* eds., 2012; P. 709-716.
8. Yu, G., Sinclair, J., Hartman, G., Bertagnoli, B. Production of chitin A by *Bacillus amiloliquifaciens* suppressing Rhizoctonia solani. *Soil Biochem*., 2002; **34**: 955-963.
9. Tendulkar, S.R., Saikumari, Y.K., Patel, V., Raghotama, S., Munshi, T., Balaram, P., Chattoo, B. Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC 98, and its effect on phytopathogen *Magnaporthe grisea*. *J. Appl. Microbiol*., 2007; **103**: 2331-2339.
10. Schister, D., Slininger, P., Behle, R., Jakson, M. Formulation of Bacillus spp. for biological control of plant diseases. *Phytopatology*., 2004; **94**: 1267-1271.
11. Tzeng, Y.M., Rao, Y.K. Tsay, K.L. Effect of cultivation conditions on spore production from *Bacillus amyloliquefaciens* B 128 and its antagonism to *Botrytis elliptica*. *J. Appl. Microbiol*., 2008; **104**(5): 1275-1282.
12. Wang, J., Liu, J., Chen, H., Yao, J. Characterization of *Fusarium graminearum* inhibitory lipopeptide from *Bacillus subtilis* IP. *Appl. Microbiol. Biotechnol*., 2007; **76**(4): 889-894.
13. Davey, M.E., O'Toole, G.A. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev*., 2000; **64**: 847-867.
14. Abriouel, H., Franz, C.M., Omar, N.B., Galvez, A. Diversity and application of *Bacillus* bacteriocins. *FEMS Microbiol. Rev.*, 2011; **35**: 201-232.
15. Azizbekyan, R.R. Using spore-forming bacteria as biological plant protection agents. *Biotechnol*., 2013; **1**: 69-77.
16. Stoytcheva, M. (Ed): Pesticides in the Modern World - Pesticides Use and Management, InTech, 2011; ðð. 520.
17. National catalog of pesticides and agrochemicals allowed to apply at Russian Federation area. M.: Agriculture Ministry, 2013; pp.708.
18. Asaturova, A.M. Physiological characteristics of promising *Bacillus* and *Pseudomonas* genus bacterial strains producing microbiological agents. *Oil Crops. ARRIOC Sci. Technol. Bull*., 2009; **2**(141): 60-66.
19. Salamatova, Yu.A., Minayeva, O.M., Akimova, Å.Å. Shelf-life efficacies of a number of liquid-state bacterial materials. *Tomsk SU Bull. Biol*. 2010; **1**(9): 20-28.
20. Pavlyushin, V.A., Tyuterev, S.L., Popova, E.V.,

Novikiva, I.I. New complex biological agents to protect vegetable crops against fungal and bacterial diseases. *Biotekhnol*., 2010; **4**: 69-80.
21. Khairullin, R.M., Lukyantsev, M.A., Urazbakhtina, N.A. Some reasons of low application efficiency of highly-antagonistic endophytic *Bacillus subtilis* strains as a base for biofungicides. *Kazan SAU Bull*., 2010; **3**(17): 146-149.
22. Monastyrsky, O.A., Dudik O.G., Yermolenko, S.A., Seleznyova, M.P. On usefulness of mass production of biopreparations for protecting grains in storage. *AGRO XXI*., 2007; **10**: 10-12.
23. Kirsanova, Ye.V. Studies in application efficiency of biological agents on grain, leguminous and cereal crops. *Oryol SAU Bull*., 2011; **5**(32): 111-116.
24. Markevichev, N.S., Chekalova, K.V., Alekseyeva, O.B. Possibilities of prolongation of shelf-life and activity period of biological agents. *Gavrish.*, 2006; **3**: 27-29.
25. Pert, S.J. (ed): Microorganisms and cells cultivation principals, M: Mir, 1978; pp. 333.
26. Mosichev, M.S. (ed): General technology of microbiological production facilities. M: Lyogkaya i pischevays promyshlennost, 1982; pp. 264.
27. Diaz de Velegas, M., Garsia, A., Seijas, B., Gonzalez Sosa, M., Castillo, R., Rodriguez, E., Iglesias, R., Gonzalez, E. Influencia del pH y la temperatura en la produccion de las fitotoxinas producidas por la *Pseudomonas aeruginosa* PSS. Evaluacion de su efectividad en el control de malezas. *Fitosanidad*., 2005; **9**(3): 41-49.
28. Chetverikov, S.P., Asabina, Ye. A., Loginov, O. N. Nutrient medium composition optimization for mass production of "Yelena" biological agent. *Bashkir Khim J*., 2006; **13**(2): P. 10-13.
29. Masliyenko, L.V. Shapiyevskaya, Ye.Yu., Asaturova, A.M. Development of production technology elements for various formulations of microbiopreparations against sunflower Phomopsis pathogen based on promising fungi antagonist strains. *Oil Crops: ARRIOC Sci. Technol. Bull*., 2007; **2**(137): 48-54.
30. Asaturova, A.M., Nadykta, V.D., Ismailov, V.Ya., Dubyaga, V.M., Tomashwvich, N.S., Zharnikova, M.D., Zhevnova, N.A., Khomyak, A.I. Studies in effect of wheat seed bacterization on plant development. *KubSAU Electron. Multidiscipl. Sci. J*., 2013; **85**(01).
31. Nadykta, V.D., Asaturova A.M. Works on environmentally safe bacterial preparation for winter wheat protection against fusarium pathogen. *Microbial biotechnol: Fundam. Appl. Asp*., 2013; **5**: 287-297.
32. Kremnyova, O.Yu., Asaturova, A.M., Volkova, G.V. Selection of bacterial strains showing antagonism to wheat leaf yellow spot pathogen. *Biotekhnol*., 2013; **5**: 54-59.
33. Asaturova, A.M., Dubyaga, V.M., 2015. Patent No. 2553518 Russian Federation, C 12 N 1/20, C 12 R 1/125, A 01 N 63/00 "Strain of bacteria *Bacillus subtilis* to produce a biological product against harmful fungi" All-Russian Research Institute of Biological Plant Protection. No. 2013151377/10; appl. 20.11.2013; publ. 20.06.2015, Bulletin, 17, 10 p.
34. Asaturova, A.M., Dubyaga, V.M., 2015. Patent No. 2552146 Russian Federation C 12 N 1/20, A 01 N 63/02, C 12 R 1/125 "Strain of bacteria *Bacillus subtilis* BZR 517 for a biological product against harmful fungi" All-Russian Research Institute of Biological Plant Protection. No. 2013151375/10; appl. 20.11.2013; publ. 10.06.2015, Bulletin, 16, 9 p.
35. Asaturova, A.M. Studies in growth kinetics of bacterial strains antagonistic to fusarium in batch cultivation. *Oil Crops: ARRIOC Sci. Technol. Bull*., 2008; **1**(138): 79-82.
36. Lysak, L.V., Dobrovolskaya, T.G., Skvortsova, I.N. (ed): Methods for assessing soil bacterial diversity and identification of soil bacteria, ed. and rev. additionally. M: Max Press, 2003; pp.120.
37. Skvortsova, I.N. (ed): Methods for identification and isolation of soil bacteria of genus Pseudomonas. M: Mosc.Univ.Press, 1981; pp.78
38. King, E.O., Ward, M.K., Raney, D.E. Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab. Ckn. Med*., 1954; **44**: 301-307.
39. Netrusov, A.I. (ed): Workshop on Microbiology: Textbook, manual. M: Academia, 2005; 608p.
40. Vaksman, Z.A. (ed): Germ Antagonism and antibiotic substances. Moscow: State Publishing House of Foreign Lit., 1947; pp. 391.
41. Yegorov, N.S. (ed): Isolation of microbial antagonists and biological methods to account for their antibiotic activity. M.: Mosc. Univ. Press, 1957; pp. 78.
42. Montealegre, J.R., Reyes, R., Perez, L.M. Selection of antagonists to be used in biological control of Rhizoctonia solani in tomato. *Electron. J. Biotechnol*., 2003; **6**(2): 116-127.
43. Guidelines for the registration tests of fungicides in agriculture / edited by Dolzhenko V.I. – M: ARIPP, 2009.
44. Lakin, G.F. (ed): Biometrics: Tutorial for spec. biol. Universities, 3rd edn. M.: Vysshaya Shkola, 1980; pp. 293.