GROWTH FEATURES OF BACTERIA *Bacillus subtilis* S2 AND S4, SELECTED FROM THE RHIZOSPHERE OF TOMATO

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Abstract. From the rhizosphere of tomatoes were isolated bacterial cultures demonstrating antifungal properties. There was carried out a series of tests after which the bacterium was identified as *Bacillus subtilis*, they were given worker numbers - S2 and S4. Bacteria have been reported to grow as colonies, stroke or continuous lawn on the surface of solid cultivation media, depending on the mode of spread (inoculation). The cultural morphology features of bacteria growth were studied on the conventional media: MPA, PDA, M9. Both cultures gave good growth on the represented media, which differ in specific features typical for this isolate. *Bacillus subtilis* S2 gave the certain well-defined colonies only on medium IPA, but on the other two media the colonies blurred, blurring the boundary of the colony. *Bacillus subtilis* S4 grew as sharply defined colonies in all of the three media. In this paper, we describe the differences in bacterial growth when inoculated by streak. Bacterial growth in the liquid media also had characteristic features: the presence of sediment and biofilm, pigmentation of cultivation media**.**

Keywords: *Bacillus subtilis*; cultivation media; cultural morphology features.

INTRODUCTION

Agroecosystem - is an artificial natural system created by human activity. These man-made ecosystems are created for specific purposes, agricultural benefits, so as increasing of ecosystems productive, improving crop yields. For this reason, the environmental sustainability of agroecosystem is low. High input agroecosystems have lower biological capacity for self‑regulation and are subjected to a threat of death from mass propagation of pests and pathogens. The one of the perspective directions of crop productivity management is increasing of the plant vitality and stamina through the use of agroecosystem native resources - soil and epiphytic organisms [10, 18, 27].

In conventional agriculture, different chemical pesticides are usually applied to control the plant pathogens. Their using leads to contamination of soil and water with residues and further accumulation of these compounds in plants which present a danger to human. An alternative to chemicals in agriculture are the biological methods of plant protection. These include the use of soil microorganisms that exhibit protective effects [2, 17, 31].

The ranges of bacteria that are most commonly extracted from the rhizosphere of different crops as antagonists of the phytopathogenic microorganism include the following taxa: *Pseudomonas, Burkhlderia, Bacillus, Seratia, Actinomycetes* [1, 5, 29].

In biological methods of plants protection from diseases the important role plays bacterial agents, in particular, bacteria of the genus *Bacillus* [1, 11, 12-14, 19]. The bacteria as producers of active substances of biopesticides favourably differ from other microorganisms. They grow well and quickly on artificial media with relatively simple chemical composition. They are easy growing in liquid media by the method of grow deep.

All of these technologies can be easily implemented in practice. The bacteria of the genus *Bacillus* show an antagonistic activity against a large spectrum of plant pathogens that infect a wide range of crops. Live-spore bacterial culture inhibits the proliferation of pathogen fungus and bacteria by their metabolic products. The use of these bioproducts for the protection of crops, which have been poorly provided with pesticides, including vegetables (cabbage, cucumber, tomato) and berries, has significant economic value [1, 8, 30].

Romanian scientists selected the isolate of the *Bacillus subtilis* from the rhizosphere of tomato, which was active against *Fusarium oxysporum* and *Alternaria sp*. From the culture fluid of this bacterium was extracted an active ingredient, which stimulated the growth of tomato and inhibited the development of the pathogen [20].

The scientists of the All-Russia Research Institute for Agricultural Microbiology RAAS isolated the *Bacillus subtilis* strain Ch-13, which shows a wide spectrum of antagonistic activities against different species of phytopathogenic fungi and bacteria. The *B. subtilis* B-13 strain produces lytic enzymes, cyanide and other antifungal metabolites, and also stimulates plant growth, producing phytohormones-auxin derivatives [4].

The treatment of the potato plants during the vegetation with the bioproduct Baksis on the base on *Bacillus subtilis* allowed to limit the development of *Phytophthora infestans* (potato late blight) on the tops at 70.0 - 75.9%, and to protect the tubers from diseases at 69.3 - 73.3% [7].

The use of the biological product Bactofit on the base of IMP-215 strain of *B. subtilis*, inhibits the growth and development of root rot in winter wheat in 4 times and the powdery mildew in 10 times. The biofungicide Fitosporin-M on the basis of culture-producing *B. subtilis*, strain 26D demonstrates biological efficacy against the *Phytophthora infestans* and *Alternaria solani*. Fitosporin-M shows biological

efficacy in postharvest storage conditions of potato tubers obtained from plots treated with this product [15].

In the study of *B. subtilis* strains TS01 and ZR02 was determined their high antifungal activity against *Alternaria solani, Botrytis cinerea, Monilia linhartiana* 869*, Phytophthora cryptogea* 759/1 *and Rhizoctonia sp.* (sterile zone was more than 37 mm in diameter). The strains were recommended for further study [28].

The liquid culture of *Bacillus subtilis*, strain BZR 336g ensured the efficiency to control the *Pyrenophora tritici-repentis (Died.)* of wheat leaves in vegetation experiment from 51.5 to 58.3% [11].

Considering the circumstance that the biological products for plant protection are less universal than chemical, it is necessary to expand the arsenal of microbial biocontrol agents, in particular bacteria, used in plant protection. Biological products can be a valuable component in integrated control programmes, one aim of which is to limit the use and impact of chemical plant protection products on non-target organisms and environment. Their importance has increased in recent years, both with possibility to reduce conventional pesticide use, and as an additional tool in pesticide resistance management strategies.

Currently, in the Republic of Moldova has not been registered any biological product based on *Bacillus* [21]. In this connection, the search and development of new biological agents that would become the producents of new biological products have become indispensable.

In this regard, the scope of the study was determination of the cultural morphology features of the growth of bacteria genus *Bacillus*, isolated from the rhizosphere of tomato, having the ability to inhibit the widespread in republic pathogenic fungi.

The aim of research was to study the growth characteristics of *Bacillus subtilis* S2 and *B. subtilis* S4 strains, isolated from the rhizosphere of tomato on different media to identify the cheaper and provide good growth of these strains, considered as a base of biological products.

MATERIAL AND METHODS

For the isolation of bacteria, the soil samples were collected at the plots of tomato cultivation in the central part of Moldova. In the areas of samples collection, no soil applications or biological treatments were carried out.

Directional selection of bacillary bacteria with antifungal properties was performed by the method of successive washes of the roots by Tepper E. et al. [26]. In the experiment 17 soil samples were used. From dug plants were taken with the help of a sterile forceps and scissors 1g of roots with sticking soil particles on them. The roots were placed in a flask with 100 ml sterile water and shaken for 2 minutes.

Further, with the help of a sterile hook roots were removed and transferred sequentially into the second, third, fourth, fifth, sixth and seventh flasks containing 100 ml of sterile water. The roots were washed in each flask during 2 min. Then, from each flask separately the 0.1 ml of soil suspension was taken with the help of a sterile pipette and applied on the cultivation media surface (PDA), further a drop must be thoroughly spread over the surface of the agar with the sterile spatula. Petri dishes were placed into the incubator at 28-30°C. The growing colonies of microorganisms were analysed after 3-5 days.

The taxonomy of selected bacteria was identified according to "Bergey's manual systematic bacteriology" [9]. Identification techniques were carried out according to the recommended methods in "Identification of soil bacteria genus Bacillus" [25].

Cultural morphological characteristics of individual colonies were performed and described on three media: PDA, MPA and M9 minimal medium [6].

RESULTS

Extensive screening of bacteria on antifungal activity showed that the greatest interest have tested *Bacillus subtilis* S2 and *B. subtilis* S4 strains, since such as different isolates of *Bacillus subtilis* S2 inhibited growth of *Alternaria alternata*, zone form 21.0 mm to 19.2 mm, while under the influence of other strains growth inhibition zone of pathogen was lower (13.3 mm, 15.5 mm). These two strains are actively retards the growth of other plant pathogens (*Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium solani*, *Fusarium oxysporum*). It was therefore of interest to study the morphological and cultural properties after cultivation on different media.

These strains were identified according to Bergey's manual, as *Bacillus subtilis* [9]. On the bases of the study it was established that this identified bacterium is a Gram-positive, aerobic, spore-forming, motile wand with rounded ends. The average sizes of bacteria in isolated cultures are as following: strain S2 – 0.8×3.3 μm (fig. 1A) and strain S4 – 1.1×3.9 μm (fig. 1B).

For the determination of the amilolytic activity of identified *Bacillus subtilis* strains was used the starch agar. Hydrolysis of starch was defined by the enlightenment zones (halo zones) around the colonies of bacteria which is clearly visible after staining with 1.2% Lugol's iodine solution (fig. 2A). The ability of bacteria to mineralize proteins (proteolytic activity) serves as a taxonomic trait, which is verified with casein hydrolysis test (hydrolysis of casein, fig. 2B) and gelatin hydrolysis test (decomposition of gelatin, fig. 2C).

On the figure 3 is clearly seen the release of oxygen by the *B. subtilis* S2, well observable by the formation of gas bubbles, indicating the bacterial cell production of the enzyme catalase (figure 3A). The culture of *B. subtilis* S2 also grows well on the 7% mineral salt medium (figure 3B).

The Voges–Proskauer Reaction was positive. With the help of this reaction was detected the presence of acetion – the chief end product of glucose decomposition in the bacterial broth culture (fig. 4A). Also, the studied bacterial strains reduced nitrates to nitrites, forming nitrate reductase, and using nitrates as a source of nitrogen. The adding of the Griess Reagent into the cultivation medium colored the cell suspension in red and pink due to the fact that in acidic medium in the presence of nitrates and aromatic amines the nitrogen compounds are formed (fig. 4B).

The tyrosine was not cleaved by the studied bacterial strains. These bacteria showed the growth on the Koser Citrate Medium that indicates the citrate utilization. Reaction of inactivation of bacteria with hen egg white was negative. The optimal growth temperature of *B. subtilis* strains - is 28-30°C.

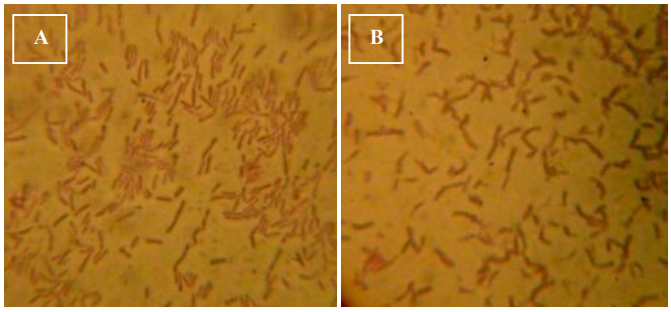

Figure 1. The appearance of cultures *Bacillus subtilis* S2 (**A**) and *Bacillus subtilis* S4 (**B**). Pictures were taken with a magnification of ×1.000 and the use of an oil immersion

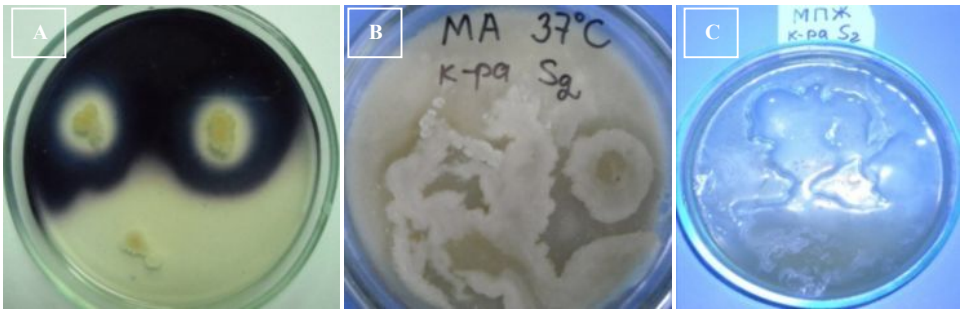

Figure 2. Amylolytic and proteolytic activity of bacteria of the genus *Bacillus*: hydrolysis of starch (**A**); hydrolysis of casein (**B**); decomposition of gelatin (**C**)

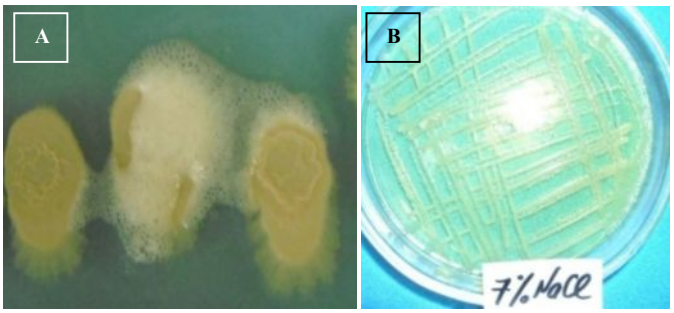

Figure 3. *Bacillus subtilis* S2 production of the catalase (**A**) and growth on the 7% mineral salt medium (**B**)

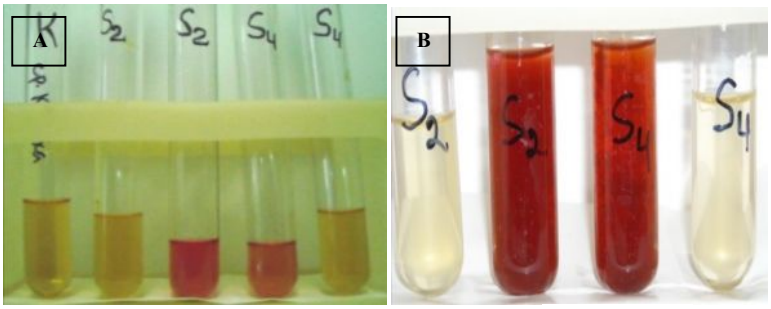

Figure 4. Chemical tests on the *Bacillus subtilis* strains S2 and S4**:** glucose decomposition - Voges–Proskauer reaction (**A**); nitrate/nitrite recovery reaction (**B**)

The next step of our studies was to investigate the bacteria growth features on solid cultivation media: PDA, MPA and M9. The results of cultural morphological features are presented in Tables 1, 2 and on figures 5-6. Comparing the growth features of two studied bacteria it was noticed that the culture of the *B. subtilis* strain S4 grew as single distinct colonies on all of the presented media. Whereas the *B. subtilis* strain S2 created well-defined colony only on MPA medium, but on PDA and M9 media - culture spreads, blurring the margins of the colonies. The number of colonies on these media could be counted only by the central areas of the colony.

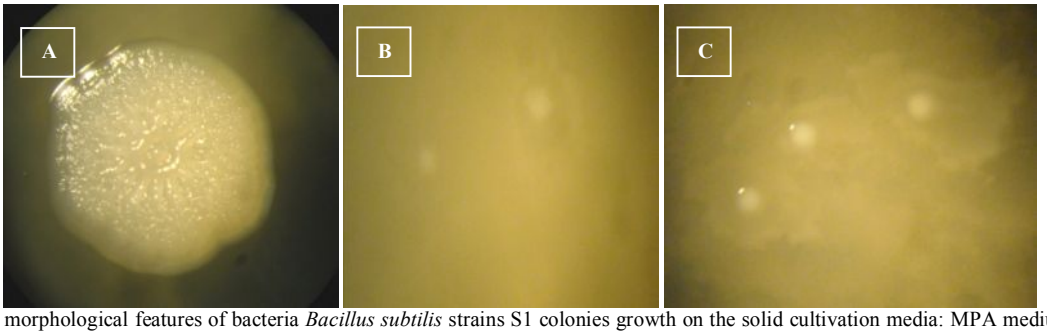

Figure 5. The morphological features of bacteria *Bacillus subtilis* strains S1 colonies growth on the solid cultivation media: MPA medium (**A**), PDA medium (**B**), M9 medium (**C**)

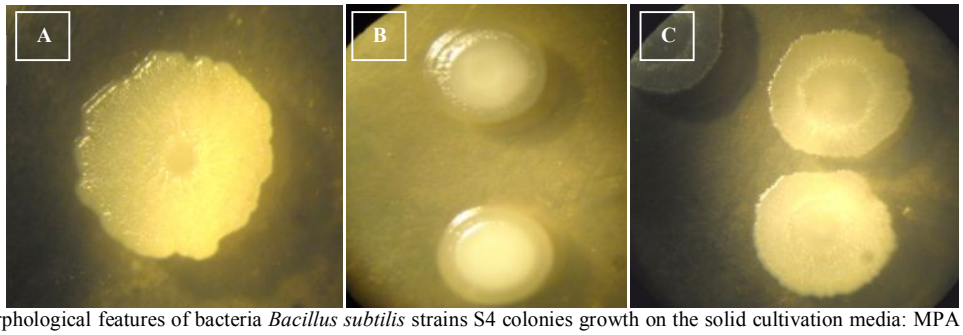

Figure 6. The morphological features of bacteria *Bacillus subtilis* strains S4 colonies growth on the solid cultivation media: MPA medium (**A**), PDA medium (**B**), M9 medium (**C**)

Table 1. Culturally morphological features of bacteria *Bacillus subtilis* strains S2 and S4 on the solid cultivation media (after cultivated 7 days)

Growth medium	Diameter of colony	Colour of colony	Growth activity
Bacillus subtilis S2			
MPA	6 mm	pale grey	8 col.
PDA	The size of the colony couldn't be determined. Colonies grow throughout the medium surface in the Petri dish.	Colourless	10 col (the amount of bacteria colonies was determined by the number of convex centre of bacteria)
M 9	19 mm × 10 mm (inside zone of bacterium)	Colourless	11 col.
Bacillus subtilis S4			
MPA	5.9 mm	Colourless	21 col.
PDA	7.5 mm	Colourless	20 col.
M 9	11 mm	Colourless	14 col.

Table 2. Description of the colonies of *Bacillus subtilis* strains S2 and S4

Growth medium	Strain	Description					
		Shape	Elevation	Margin	Structure	Surface	Consistency
MPA	*B. subtilis*-S2	curled	crater form	entire	irregular	wrinkled (or shriveled)	viscid (sticks to loop, completely removed from the agar)
	B. subtilis-S4	circular with scalloped margin	crater form	wavy	coarse grainy	wrinkled, furrowed	dense, soft, easily removed from the agar
PDA	*B. subtilis*-S2	irregular	undulate	vague	smooth	smooth	soft, easily removed from the agar
	B. subtilis-S4	circular	convex	entire	coarse grainy	smooth, folded on the edge	soft, easily removed from the agar
M9	*B. subtilis*-S2	irregular	bent	wavy	fine grainy	smooth	soft, easily removed from the agar
	B. subtilis-S4	circular with scalloped margin	bent	wavy	irregular	smooth, wrinkled on the periphery	soft, easily removed from the agar

It is known that microorganisms, developing on the surface of solid cultivation media, can grow as a colony, as a streak or lawn of bacterial growth, depending on the mode of spread. Thus, on the figures 7 and 8 are presented differences in the growth of *B. subtilis* S2 and *B. subtilis* S4 inoculated by streak. The streak of the *B. subtilis* S2 on the PDA cultivation medium was vague, with the dense inside zone of streak, wrinkled on the periphery. The streak of the *B. subtilis* S4 was distinct, hearty, and solid with wavy edge (fig. 7, 8A).

On MPA medium the both bacterial cultures are growing poorer. The streak of the *B. subtilis* S2 consists from a chain of clear isolated colonies, while the streak of *B. subtilis* S4 is moderate, with tightly packed colonies of a few streak chains (fig. 7b, 8b). On M9 medium the streak of the *B. subtilis* S2 is very weak, barely noticeable, but the streak of the *B. subtilis* S4 is moderate, entire, with a wavy margin, mucosal (fig. 7C, 8C).

The growth of microorganisms in a liquid cultivation medium is more uniform. It is accompanied by opacification of the medium, formation of a biofilm or followed by bacterial sedimentation. Frequently the growth of microorganisms is followed by staining of medium.

The growth of bacteria *B. subtilis* S2 in the potato dextrose broth (PDB medium) is accompanied by the formation of a thin folded microbial biofilm, abundant friable flocculent sediment, which is predomenantly located at the bottom of the whole (fig. 9A). The growth of bacteria *B. subtilis* S4 in liquid culture is also followed by the formation of a thin and smooth biofilm (fig. 10).

As shown in the submitted figures, the part of sediment is presented on the bottom in the form of scant and homogeneous mass, and other part - floats in the thickness of the medium, occupying the 2/3 volume of liquid. The area of the cultivation medium between the biofilm and bacterial suspension is transparent. The titer of cells in the cultures after 2 days of growing in the PDB medium reached: for *B. subtilis* S2 – 2.1×10^9 CFU / ml; *B. subtilis* S4 – 1.0×10^9 CFU / ml; after three days of cultivation the titer reached: for *B. subtilis* S2 -1.8×10^{10} CFU / ml and for *B. subtilis* S4 – 3.3×10^9 CFU / ml.

Bacterial growth in M9 liquid medium was also characterized by the pigmentation of mediul (fig. 9B). The medium M9, where was growing the *B. subtilis* S2 acquired a faint pale pink hue, and where was growing the *B. subtilis* S4 - the pale gray-green color was given to this medium.

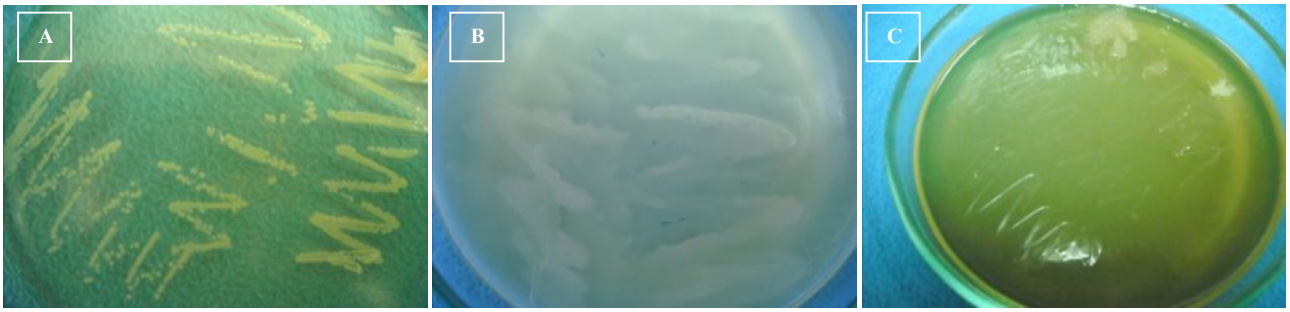

Figure 7. Streaking growth of bacterial culture *B. subtilis* S2 on the solid cultivation media: MPA medium (**A**), PDA medium (**B**), M9 medium (**C**)

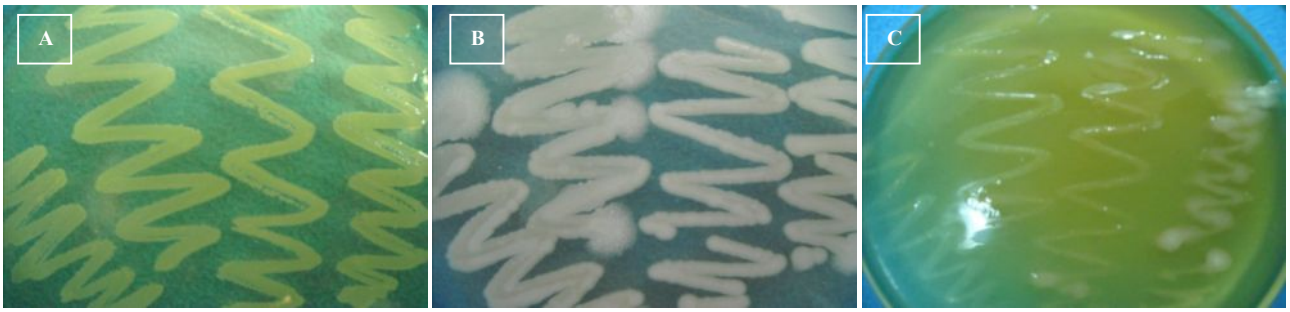

Figure 8. Streaking growth of bacterial culture *B. subtilis* S4 on the solid cultivation media: MPA medium (**A**), PDA medium (**B**), M9 medium (**C**)

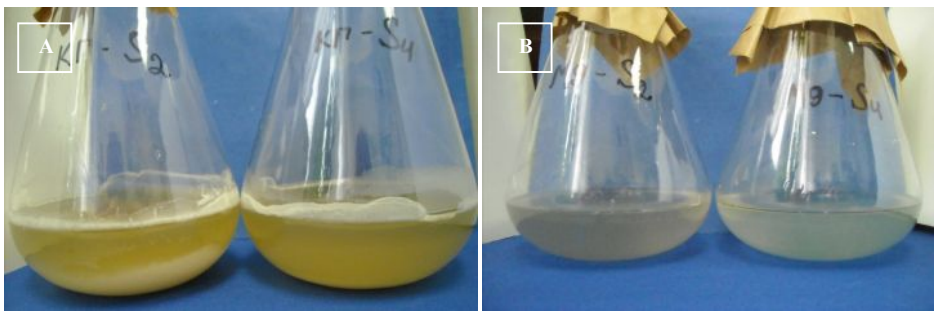

Figure 9. Growth features of bacteria genus *Bacillus* in a liquid cultivation medium: in the PDB medium (**A**); in the M9 medium (**B**)

The *B. subtilis* S2 growth in the cultivation medium resulted the very slight turbidity of the medium, the formation of a thin barely visible biofilm and a homogeneous light loose sediment. The *B. subtilis* S4 growth also is accompanied by a slight opacification of the medium, wherein the biofilm is not formed and the sediment is lean, loose, flaky.

DISCUSSIONS

The *Bacillus* is a genus of several dozen species of aerobic Gram-positive, rod-shaped, spore forming bacteria. Members of the genus *Bacillus* are primarily aerobic soil inhabitants that are non-infectious. They are also found in air, on plants, foods as well as in the gastro-intestinal tract of insects and animals. *Bacillus subtilis* can also be found in the human body, mostly on the skin or in the intestinal tract. However it is very rare for this bacteria to colonize on the human body.

Bacillus subtilis and close relatives are not considered pathogenic or toxic and are not a disease causing agents, don't synthesize endotoxins and other toxic substances. Many of them have the status of non-harmful organisms (GRAS, generally regarded as safe), assigned to them by the Office of monitoring quality of food and medicines USA (FDA) [22, 23].

Clarification of the taxonomic position of the microorganisms, pure culture isolated, often is a precondition for further work with them.

The most significant growth features of microorganisms on solid cultivation medium is the colony character. Different bacterial cultures behave differently when spreading on cultivation media. In our study the bacterial strain *Bacillus subtilis* S4 have presented the same growth characteristics on the all three cultivation media, giving individual clear colonies, while the strain *Bacillus subtilis* S2 - only on the MPA medium, which is in accord with data that showing that *Bacillus subtilis* n3 on the medium MPA is also growth by individual distinct colonies [3].

This suggests that for determination the titer of the cells of the *Bacillus subtilis* S2 it is desirable to use MPA medium, because the bacterial colonies grow clear with a well-defined edges, while on the other two media - PDA and M9 - colonies spread, the same pattern of growth of culture *Bacillus subtilis* 1719 - vague colony on potato-glycerol medium was observed and by Russian researchers [16]. It can be assumed that this is because the culture was grown on complex organic medium of undetermined composition (presence in the medium of potato decoction) which can lead to errors in counting of the number of bacterial colonies [6].

We offered to inoculat the selected strain of *Bacillus subtilis* on PDA cultivation medium for best storage. It was obtained a good strong growth and biomass accumulation of both bacterial strains on this medium. This is consistent with the classic conditions for storage of this type of cultures in accordance to Egorov [5]. The results of our studies on the growth characteristics of the strains are consistent with the data presented by Borozdina I.B. (2011), that in her experiments also showed that the culture of the genus *Bacillus* studied it well sporulate on a potato medium, which is proposed by other researchers as the best to store these bacteria [3, 6, 24].

Preliminary experiments on the selection media to ensure high titer cultures revealed that cultures such highest titer observed on the medium MPA (*B. subtilis* S2 – 4.1×10^9 CFU / ml, *B. subtilis* S4 – 9.4×10^8 CFU / ml), on PDA medium (*B. subtilis* S2 – 1.2×10^9 CFU / ml, *B. subtilis* S4 – 3.3×10^9 CFU / ml), on M9 medium titer was lower (*B. subtilis* S2 – 3.0×10^8 CFU / ml, *B. subtilis* S4 – 4.3×10^7 CFU / ml). Given that the media of the PDA and the MPA have complex composition and are quite expensive, it is appropriate to continue to use the M9 medium that is easy to prepare and low cost for the development of new media contribute to maintaining and increasing antifungal properties of the studied strains.

PDA and MPA are the natural media, whereas M9 – is a synthetic medium. As are known the natural media are not easy to use for production of culture, because they are very expensive and required the using of food products and a long time for their preparation. The synthetic medium M9 is cheaper and easier to performance. Thus, the M9 medium can serve as a basis for further development of the best cultivation media for the studied bacteria.

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