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# CULTURAL MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF *BACILLUS SUBTILIS* STRAINS

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**SUMMARY**

A decrease in therapeutic effect of some live lacto- and bifidobacteria-based drugs for veterinary use has been observed for the last 20 years that urges scientists to search for new microorganisms possessing probiotic properties. Many studies in this field are focused on *Bacillus subtilis* that is widespread in the environment and non-pathogenic for animals and humans. Results of tests of *Bacillus subtilis* for its biological properties and antagonistic activity aimed at optimization of methodical approaches for detection of strain with the highest antagonistic effect on some opportunistic microorganisms and their further use as probiotics are described. Cultural morphological and biochemical characteristics of the tested strains conformed to the species characteristics of *Bacillus subtilis.* Tested strains were non-pathogenic for white mice. Tests showed that spore biomass could be prepared both in liquid and on solid nutrient media. Methodically, spore biomass preparation in liquid nutrient medium is preferable. The tests showed that spores emerged from anabiosis non-uniformly and it depended on original seed spore storage period. Spore cultures stored less than one year emerged from anabiosis more quickly. It was found that the spores formed more readily when the cultures were aerated with oxygen as well as that lag-phase culture medium had a stimulating effect on *Bacillus subtilis* spore germination. *Bacillus subtilis* strains were found to have antagonistic effect on *Escherichia coli, Salmonella* and *Staphylococcus*. Area of growth inhibition of the said bacteria was 15–20 mm. Tested *Bacillus subtilis* strains could be proposed for use as probiotics.

Key words: probiotics *Bacillus subtilis*, cultural and morphological properties, biochemical properties, spore formation, antagonistic activity.

## INTRODUCTION

One of the characteristics of microorganism relations in nature is their antagonism, i.e. inhibition of one bacteria by another. These bacterial characteristics found practical use in veterinary medicine as probiotics.

It should be noted that wide use of different probiotic agents based on live lacto and bifidobacteria in veterinary medicine and healthcare within the last decade has led to decrease in therapeutic effect and nudged the scientists towards search for new, more effective microorganisms with probiotic characteristics. Some of the most suitable biological objects are some types of spore-forming bacteria, and primarily, *Bacillus subtilis* [2, 3]. In the present time more than 50 different spore probiotics have been developed in the world. Due to co-development of *Bacillus subtilis* with other bacteria species bacilli antagonistic activity determines survival of microorganisms in case of their interaction in microbial communities. *Bacillus subtilis* form antibiotic substances and due to that have bactericidal and bacteriostatic effect on other microorganisms. Hay bacillus can effectively inhibit growth of different opportunistic bacteria such as *E. coli*, streptococci, staphylococci and others. Antagonistic action of *Bacillus subtilis* on a considerable number of microorganisms opens great opportunities for development of new therapeutic and preventive agents for animals on their basis. It should be noted that these positive probiotic characteristics of *Bacillus subtilis* are not common to each strain [6, 8]. Probiotic agents of many trademarks are based on different strains of *Bacillus subtilis*. The mechanism of probiotic action of these agents based on different bacilli is described in publications [1, 7].

This paper was aimed at studies of biological properties of several *Bacillus subtilis* strains, their antagonistic action, spore-forming process and spore biomass preparation as one of the stages of activities aimed at search of a candidate for a probiotic agent.

## MATERIALS AND METHODS

*Microorganism strains*. *Bacillus subtilis* strain No. 1232, obtained from the Museum of microbiology, virology, epidemiology, veterinary and sanitary examination of the Ulyanovsk State Agrarian University after P. A. Stolypin was used in the investigation. Reference strain ATSS 6633 was used as the control strain.

*Nutrition media.* Meat peptone broth (MPB) was used according to GOST 20730-75. Liquid sporulation and growth medium [5], 2% meat peptone agar (MPA), 2% potato peptone agar, Giss media as well as demineralized water were also used in the investigation.

*Bacteria morphology and tinctorial properties* were studied using a phase-contrast microscope as well as Gram staining of vegetative cells. Bacteria motility was determined by the wet mount using 5, 16 and 18 hour broth cultures.

*Culture properties* were studied basing on the character of growth in liquid and solid media.

*Biochemical properties* of the cultures not older than 18–20 hours, grown in solid medium and Giss media were determined using indicator paper test-kits for microorganism identification and bacteriological analyzer VITEK® 2 Compact using GN and GP identification cards as well as cards for detection of antimicrobial sensitivity of microorganisms.

Bacteria inoculated-Giss media was left for two days at 35–37 °C. The presence and absence of carbohydrate fermentation was determined by the medium colour change.

*Hemolytic efficiency* of bacteria was studied using meat peptone agar containing 5% of the freshly collected defibrinated ram blood.

*Indole and hydrogen sulphide formation* was determined using the indicator paper placed into the tube with meat peptone broth.

*Nitrate reduction* was determined after the culture growth in the nitrate broth ($KNO_3$) within 48 hours. 1 $cm^3$ of the reagent (equal solution of 10% pure sulphuric acid and solution containing 1% soluble starch and 0.5% potassium iodide) were added to each tube with inoculated nitrate broth.

*Casein hydrolysis* was studied using milk agar. The culture was inoculated using streaking, then it was incubated in the thermostat at 37 °C and observed for clear transparent zones.

*Starch hydrolysis* was tested in potato peptone agar. Petri dishes with the inoculated agar were flooded with Lugol's solution after 48 hour incubation at 37 °C. Clear zones around the inoculations were indicative of starch hydrolysis.

When growing *Bacillus subtilis* bacteria in nutrient broth growth intensity and medium turbidity were observed in liquid nutrient medium.

*Growth purity control* was performed by microscopy of stained smears. Light microscopy of Gram-stained smears or smears stained with fuchsine solution (for spores), was performed using MBI-15 microscope with magnification of ×900.

*Concentration of viable spores was calculated according to the formula:*

$$C = \frac{\overline{N}_n + \overline{N}_{n+1}}{1{,}1} \times \frac{1}{V} \times 10^n,$$

where $C$ –number of viable spores in 1 $cm^3$ spore suspension;

$\overline{N}_n$ – arithmetic mean of colonies grown in dishes inoculated with $10^{-n}$ bacteria dilution;

$\overline{N}_{n+1}$ – arithmetic mean of colonies grown in dishes inoculated with $10^{-(n+1)}$ bacteria dilution;

1.1 – constant coefficient;

$V$ – volume of spore suspension inoculated in dishes;

$10^n$ – dilution of spore suspension used for determination of the viable spore quantity.

*Antagonistic activity of Bacillus subtilis* was assessed *in vitro* using a thin agar layer method based on diffusion of *Bacillus spp.*-produced antibiotics into agar medium containing test-culture and inhibition of its growth. *Escherichia coli*, *Salmonella cholerae suis,* strain No. 370, *Staphylococcus aureus* served as test-cultures. To assess *Bacillus subtilis* antagonist activity 0.7% meat peptone agar was layered onto Petri dishes with 2% meat peptone agar. Beforehand, the agar was melted, cooled upto 46–48 °C, inoculated with the test-culture and after mixing it was added to the 2% meat peptone agar evenly distributing over the surface. After 16–18 hours of growth at 35–37 °C, a drop of the *Bacillus subtilis* strain under study was applied to the surface of a semi-liquid agar with a inoculating loop. The results were recorded after 16–18 hours of incubation at 35–37 °C basing on the size of the zones with no test-culture growth around the tested *Bacillus subtilis* strain.

*Pathogenic properties* were determined usinig outbred white mice weighing 16 g, which were intraperitoneally injected with 0.5 $cm^3$ of *Bacillus subtilis* broth culture with a concentration of $\times 10^9$ cells per $cm^3$. The animals were observed for 10 days.

All experiments on animals were carried out in strict accordance with interstate standards for the maintenance and care of laboratory animals GOST 33216-2014 and GOST 33215-2014 adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament and the Council of the European Union of September 22, 2012 On protection of animals used for scientific purposes.

## RESULTS AND DISCUSSION

The first series of experiments was performed to determine the cultural, morphological and biochemical properties of *Bacillus subtilis*. For this purpose, the tested bacterial strains in a volume of 0.1–0.2 $cm^3$ were inoculated in solid and liquid nutrient media, as well as Giss media. The inoculum was evenly distributed over the MPA surface by rocking, Petri dishes were dried for 30–40 min at room temperature, transferred to a thermostat, and incubated upside down at 35–37 °C for 20–24 hours. Inoculations into a liquid nutrient medium were cultivated in the same mode.

Culture growth, typical of *Bacillus subtilis*, was observed in inoculations into MPB, accompanied by clouding of the medium and the formation of a hard-to-break film of grayish-white color on the surface of the broth.

On the MPA surface, after a one-day incubation, grayish-white, uneven colonies with uneven edges (Fig. 1) of a viscous consistency were observed. Gram-positive chains and rods with a diameter of about 0.6 μm and a length of 3–5 μm were observed in Gram-stained smears (Fig. 2).

In drugs prepared using a crushed drop method cell were mobile due to their flagella.

Studying biochemical properties of the tested strains showed that bacteria ferment sucrose, glucose, manit, salicin, esculin, fructose and do not ferment lactose, maltose, rhamnose, dulcite, inosite, sorbite, galactose, raffinose.

Bacteria don't form indole but they form hydrogen sulphide, hydrolyze starch, casein, but don't hydrolyze tyrosine and don't hemolyze ram erythrocytes, reduce nitrates and produce catalase.

To confirm the capability of the cultures under the study to form spores 0.3–0.5 $cm^3$ of broth culture were ino-

culated to Petri dishes with potato peptone agar evenly distributing the seeding material over the agar surface by rocking. The inoculated material was dried using a thermostat at 35–37 °C for 20–30 min, turned upside down and incubated for 6–7 days. Random microscopy of smears from cultures grown in the "crushed drop" preparation and stained with fuchsine (Fig. 3) demonstrated presence of 0.8×0.6 μm oval spores located in the center of the cell.

*Bacillus subtilis* antagonistic activity against *Escherichia coli*, *Salmonella cholerae suis* (Strain No. 370), *Staphylococcus aureus* was tested on nutrient media. The performed tests demonstrated the presence of test-culture inhibition zones, 15–20 mm.

The tested strains of *Bacillus subtilis* were non-pathogenic for white mice.

The second series of experiments was aimed at spore biomass preparation and determination of their viability. Spore biomass accumulated both on a solid and on a liquid nutrient medium.

For spore preparation a one-day 0.3–0.5 $cm^3$ *Bacillus subtilis* broth culture was inoculated on Petri dishes with potato peptone broth. The inoculated bacteria were incubated for 6–7 days. Spore formation percent was determined by random microscopy of smears from the dishes using "crushed drop" preparation. When examining 3–5 smear fields and detecting not more than 15–27 vegetative forms per 80–100 spores, the spores were washed off the potato peptone agar with demineralized water. Lysis of remaining *Bacillus subtilis* vegetative cells was performed by heating the spore suspension in a water bath at 80 °C for 30–40 min. The spores were washed twice to remove the nutrient medium residues by centrifuging at 4–5 thousand RPM for 20–30 min and for subsequent resuspension.

The number of viable spores was determined using MPA. For that purpose 15–20 $cm^3$ of melted and chilled (to 45–50 °C) MPA were filled into sterile Petri dishes. The tested spore culture was thoroughly shaken and its 10-fold dilutions ($10^{-1}$–$10^{-8}$) were prepared using sterile demineralized water with Tween-80 (two Tween drops per 100 $cm^3$ of water). An individual pipette was used for each dilution. The dilutions were left for 15–20 min at room temperature.

After thorough mixing the material from two last dilutions of spore suspensions ($10^{-7}$ and $10^{-8}$), (staring from the last one) was inoculated in Petri dishes with MPA using sterile micropipette, an individual pipette for each dilution. 0.1 $cm^3$ of the suspension from each dilution was inoculated into three Petri dishes. After the seeding material have been evenly distributed over the MPA by rocking Petri dishes were dried for 30–40 min at room temperature, transferred to the thermostat, placed upside down and incubated at 35–37 °C for 20–24 min.

At the end of the specified time the colonies grown on MPA were counted in each Petri dish, Then, the arithmetic mean of colonies of each spore suspension dilution was calculated.

For *Bacillus subtilis* spore biomass preparation on liquid nutrient medium cultivation was performed in 2 litre flasks containing 300 ml of liquid nutrient sporulation medium at 35–37 °C using a shaker (120 RPM). In 24 hours of cultivation the spores were collected and washed with sterile demineralized water by centrifuging at 10,000 g for 15 min.

The number of viable cells was determined by calculation of colonies formed after inoculation of spore suspensions on MPA.

*Fig. 1. Morphology of Bacillus subtilis (Strain No. 1232) colonies in 24 hours incubation on MPA (×10)*

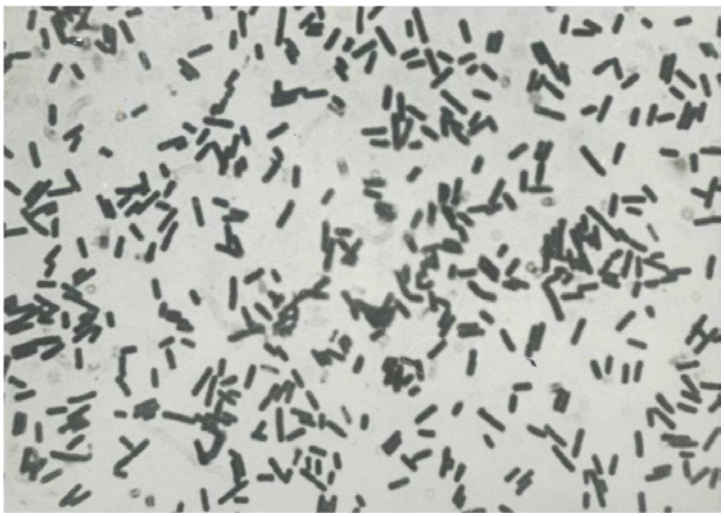

*Fig. 2. Morphology of Bacillus subtilis (Strain No. 1232) cells in 24 hours of incubation on MPA (Gram staining, ×900)*

Microscopic examination of *Bacillus subtilis* spore culture germination demonstrated that they emerged from anabiosis non-uniformly. Non-uniform spore germination changed depending on the period of original spore seed storage. Spore culture stored less that one year germinated faster.

Taking into account that bacilla spore germination process is considerably influenced by the amount of oxygen in the nutrient medium [4], the nutrient medium of the same contents was used in all series of experiments. Cultivation with shuttling provided 75–85% oxygen aeration of the culture.

The applied aeration mode guaranteed 80–95% spore initiation 10 min after they have been inoculated into a nutrient medium. Apparently, presence of oxygen in the nutrient medium contributes to a more rapid flow of processes in spores, leading to coming out of anabiosis.

In the final part of the experiments, the effect of the culture fluid on the spore germination process was studied. Stationary cultivation was performed. Germinating spores in the control and test samples were counted every 15 min from the moment of their inoculation into the nutrient

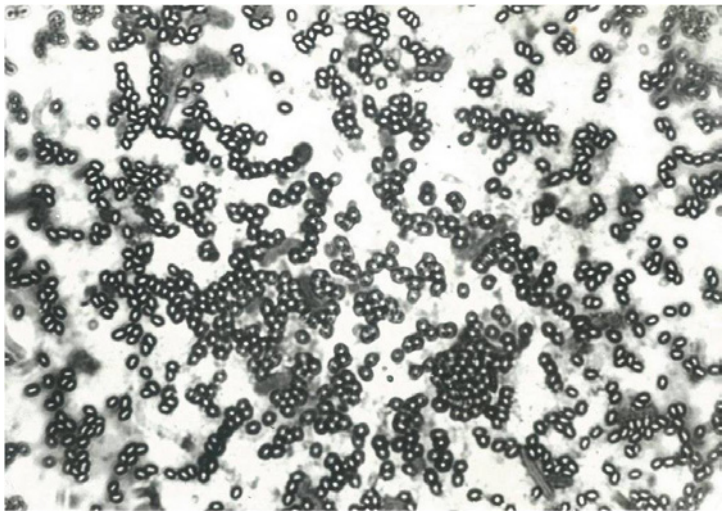

*Fig. 3. Spore-forming Bacillus subtilis, strain No. 1232, in 4 days of incubation on potato peptone agar (fuchsine staining, ×900)*

medium. The evaluation was performed by a microscopic method and by the change in optical density. The tests showed that in 45 min the number of germinated spores increased to 90% compared to 25–30% in control samples. During the experiments the stimulating effect of the culture fluid from the lag phase on the germination process was revealed. The same phenomenon was observed when seeding the treated dormant spores *of Bacillus subtilis* on solid nutrient media. At the same time, an 50–60% increase in the number of grown colonies was observed in comparison with control samples. Apparently, in this case, the initiation of superdormant spores, which do not germinate without prior activation, occurs.

The results obtained are consistent with the published data indicating the need for thermoactivation for germination of a part of *Bacillus subtilis* spore population being in a state of deep anabiosis [4].

It can be assumed that stimulation is manifested not in the reduction of the time of the main germination, but in an increase in the number of spores that have come out of anabiosis and germinating spores.

## CONCLUSION

The cultural, morphological and biochemical characteristics of *Bacillus subtilis* strains No. 1232 and ATCC 6633 conform with species characteristics. Methods used in the research allow preparation of *Bacillus subtilis* spore material both in liquid and solid media.

The studied strain of *Bacillus subtilis* No. 1232 showed antagonistic activity against all three species of the tested bacteria: *Escherichia coli*, *Salmonella cholerae suis* and *Staphylococcus aureus* – and can be proposed as a candidate for use as a probiotic.

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