Determination of Taxonomic Affiliation and Assessment of Biotechnological Potential of an Indigenous Strain of Gram-Positive Spore-Forming Bacteria

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Abstract. The search for new effective strains-producers of enzymes and biologically active substances for the development of new environmentally friendly plant protection products against phytopathogens is a very urgent task for the greening of agriculture. In our work, we used active strains of gram-positive spore-forming bacteria isolated from soil near the roadside zone of the Belgorod region to assess their antagonistic activity to phytopathogens and biotechnological potential. In the course of molecular genetic studies of the 16S rRNA gene of the isolated native strain, a complete similarity of the target with *Bacillus subtilis* was revealed. When studying morphological and cultural characteristics, it was determined that the cells of the target strain are rod-shaped, arranged singly or in pairs.The conditions of cultivation of the bacterium were revealed, which shows high growth in the composition of the medium containing 3% peptone for 20 hours, the specific increase in biomass exceeded 96%. When evaluating the antagonistic activity, it was revealed that the isolated strain suppresses the growth of cultures of *Escherichia coli* O157:H7, *Aspergillus unguis* VKM F-1754 and bacteria of the genus *Ralstonia*.

1 Introduction

Agriculture has a significant impact on the environment. The negative effect of agriculture is that a decrease in soil fertility leads to water and wind erosion of soils, loss of organic matter, water-retaining capacity of soils and their biological activity. Agriculture is the largest source of water pollution, including salinization, fertilizers (nitrates, phosphorus), pesticides and organic fertilizers. Poor groundwater quality has a significant impact on agricultural production, drinking water quality and fish production. Another serious environmental problem is that currently about 400 species of harmful insects and about 70 species of phytopathogenic fungi have acquired resistance to one or more types of pesticides [1]. In this regard, there is a need to revise modern approaches to land use, the development of environmentally friendly agricultural technologies, including the search for

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new biological products based on non-pathogenic soil bacteria living in association with the roots of higher plants.

As of 2020, a large increase in the acreage of oilseeds of plants has been established in the Belgorod region [2]. Figure 1 shows that the increase from 2000 to 2020 increased by 3.5 times. All seeds of oilseeds have shells, which are separated during the processing of seeds for oil, making up the main type of waste from oil production - cake and meal. The cake is obtained by extracting oil by pressing, it contains up to 10% fat.

Currently, the largest sector of the biotechnological industry is the production and use of enzymes of microbial origin [3]. Enzymatic catalysis occupies a special place in the field of biotechnology, due to the variety and specific properties of enzymes. The most important enzymes for the chemical, food, pharmaceutical and fuel industries are hydrolases (lipases and esterases), which are used in hydrolysis and esterification reactions, especially in the immobilized form [4]. Studies with various microorganisms and substrates for the production of lipases in liquid media can contribute to the search for optimal conditions for obtaining high enzyme yields and, thus, reducing production costs on an industrial scale.

The aim of this study: determine the taxonomic affiliation of an indigenous strain of gram-positive spore-forming bacteria isolated from the soil near the roadside zone of the Belgorod region and to assess its biotechnological potential for expanding the range of producing strains that can become the basis for the development of new technologies.

2 Material and methods

From the soil of the Belgorod region by the method of serial dilutions, on a dense mineral nutrient medium with Na-carboxymethyl cellulose as a growth substrate (composition (g/l): Na-carboxymethylcellulose - 5 KNO3 - 0.4, KH2PO4 - 0.06, Na2HPO4 - 0.14, MgSO4 - 0.08, microbiological agar - 20) and at a temperature of 35 ° C, a strain designated as KE-1 was isolated, which showed lipase activity [5]. Soil samples were taken from roadside land in the village of Klinovy in the Prokhorovsky district, studies were conducted from 2021 to 2022.

The cultural and morphological properties of the isolated culture were studied when cultured in a 3% peptone agar medium (composition: 3% peptone, 2% microbiological agar) using a stereoscopic microscope MICROMED II (Russia).

The Gram reaction and the ability to form capsules (according to Peshkov) were evaluated by the standard method [5].

The physiological and biochemical properties of the isolates were evaluated by growing crops on nutrient media with a different substrate for growth. Each sample was grown for 24 hours at a temperature of 25 ℃.

The ratio to oxygen was assessed by the nature of the growth in the thickness of the medium when the culture was sown in a column with agar by injection [5].

The optimal temperature range for the growth of the isolate was chosen when cultivating the strain in a liquid peptone nutrient medium at different temperatures. After 20 hours, photometric growth of the culture was observed in three repetitions at a wavelength of 600 nm using a UV-1900i spectrophotometer (Shimadzu, Japan). The resulting optical blocks were used to calculate the specific gain parameter by the previously described method [12]

In those variants where the specific increase was the smallest, the growth of the culture was considered insignificant relative to the more effective ones.

The spectrum of compounds used as a growth substrate by the KE1 strain was determined in a liquid mineral medium. For this purpose, a medium and a 1% solution of sucrose, D-glucose, lactose, D-fructose, lead acetic acid, tartaric acid, sodium citrate, potassium-sodium tartrate, benzoic acid, sorbic acid, sodium benzoate, 0.5% solution of

salicylic acid and sodium acetate were prepared. After autoclaving, 1 ml of the solution was introduced into the mineral medium and the initial suspension of the isolate was passed (in a volume of 100 µl in 10 ml of the medium). The ability to use organic compounds as a growth substrate was judged by the turbidity of the medium. An unseeded nutrient medium was used as a control variant.

To isolate chromosomal DNA, a single colony of strain KE1 was seeded into a nutrient medium (3% peptone) and cultured at 30°C for 12 hours. Further, cells were deposited from 1 ml of culture by centrifugation at 10000g for 3 minutes. DNA was isolated from the resulting cellular sediment using the GeneJET Genomic DNA Purification Kit (Thermo Scientific). To amplify the 16S rRNA sequence with a size of about 1.5 kb, three pairs of universal primers complementary to the conservative regions of the gene were used. PCR was performed in 25 µl of a mixture containing 100 ng of genomic DNA, 10x PCR buffer (67 mM tris-HCl (pH 8.8), 16 mM (NH4)2SO4, 5 mM β-mercaptoethanol, 7 mM EDTA, 3 mM MgCl2), 0.25 mM dNTP, 0.5 µm of each primer, 1 unit of SynTaq DNA polymerase T+ (Synthol). Amplification was performed on a Veriti (Applied Biosystems) thermal cycler. The amplification conditions are presented in Table 1.

Table 1. Conditions of polymerase chain reaction

№	The sequence of primers	Amplification mode	Size PCR product	Amplified 16S rRNA site
1.	27F/5'-AGAGTTTGATCCTGGCTCAG-3' 534R/5'-ATTACCGCGGCTGCTGG-3'	95°C – 3 min; 35 cycles: 94°C - 30°C; 60°C - 30°C; 72°C – 40; 72°C - 5 min.	527 bp	V1-V3 (Haakensen, 2008)
2.	SSU-642-F/5'-HAATHYGTGCCAGCAGC-3' SSU-1445-R/5'-GTCRTCCYDCCTTCCTC-3'	95°C - 3 min; 35 cycles: 94°C - 1 min; 55°C - 1 min; 72°C - 1 min; 72°C - 5 min..	686 bp	V4-V6 (Patyka et al. 2009)
3.	1099F/5'-CGYAACGAGCGCAACCC-3' 1492R/5'-GNTACCTTGTTACGACTT-3'	95°C - 3 min; 35 cycles: 94°C - 30 C; 55°C - 40 C; 72°C - 30 C; 72°C - 5 min.	404 bp	V7-V8 (Godon, 1997)

After amplification, the quality of the target amplicons was tested in 2% agarose gel. Amplicons were purified from PCR mixture products using ExoSAP-IT reagent (Applied Biosystems). The sequencing reaction was carried out with direct and reverse primers using a set of reagents BigDye® Terminator v3.1 Sequencing kit (Applied Biosystems) according to the protocol. Purification of the products of the sequence reaction was carried out by precipitation of 96% ethanol with 125mM EDTA. Electrophoresis of the products of the sequence reaction was carried out on an automatic capillary DNA analyzer ABI PRISM 3500 (Applied Biosystems, USA), using capillaries 50 cm long and a polymer matrix POP-7TM. The data obtained was initially processed in Sequencing Analysis® Software 6 (Applied Biosystems). The alignment of forward and reverse sequences was carried out in the BioEdit V.7.0.0 program [7].

The search for homologous sequences was carried out in the GenBank database using the BLAST option [8].

The selection of the optimal medium for the cultivation of the microorganism was carried out as follows: the following substances were added to 4 types of different nutrient media: the first is with the addition of 3% peptone and 2% agar, the second one is mineral, the third – with the addition of 3% peptone, 2% agar, 1% Na-carboxymethylcellulose (Na-KMC), the fourth – with the addition of 3% peptone, 2% agar, 1% starch.

For 20 hours, the growth of the culture was observed photometrically in three repetitions at a wavelength of 600 nm using a UV-1900i spectrophotometer (Shimadzu, Japan). The resulting optical blocks were used to calculate the specific gain parameter by the previously described method.

The antagonistic potential of the isolated KE1 strain against *Escherichia coli* O157:H7 was evaluated by the agar disks method [5]. The daily culture under study was passioned with a "lawn" of 3% peptone with agar and incubated at 35 ° C for 24 hours. In agar with the grown KE1 bacterium, disks were cut out with a sterile cork drill and placed on a newly prepared "lawn" of *E. coli* O157:H7. The crops were incubated at 35 °C for 48 hours. The antagonistic potential of KE1 was judged by the growth suppression zone of *E. coli* O157:H7 around the agar disk.

Antagonistic properties of the native strain in relation to bacteria of the genus *Ralstonia* and *Escherichia coli* O157:H7 were determined by co-cultivation in a liquid nutrient medium - 3% peptone. Antagonistic activity was judged by the change in the number of test cultures (CFU/ml) in comparison with pure culture (without a potential antagonist). To do this, cultures (researched culture and test culture) were sown on a Petri dish with agarized nutrient medium (3% peptone) by the method of serial dilutions "lawn" [5] in the amount of 100 µl per cup, incubated separately and jointly, every 24 hours. After a day, the grown colonies were counted and the total number was summed up using the formula.

$$N = \frac{a \cdot 10^n}{v}, \quad (1)$$

where: a - the arithmetic mean; 10^n – the breeding; V – the volume of the aliquot, ml.

The value of colony-forming units was used to calculate the degree of inhibition (the inhibition rate, IR) of the test culture by an isolate - a potential antagonist according to the formula [9]:

$$IR = \left(\frac{\lg N_K - \lg N_O}{\lg N_K}\right) \cdot 100\% , \quad (2)$$

where lg N_k is the number of colony-forming (CFU/ml) culture units in the control variant at the end of incubation, and lg N_o - the number of the prototype cultivated jointly.

To assess the antagonistic potential of the native strain against the mold fungus *Aspergillus unguis* VCM F-1754, the method of co-cultivation [10] was used on an agarized Saburo nutrient medium (composition (g/l): glucose - 40.0; peptone - 10.0; yeast extract - 5; agar - 18.0). Meanwhile, the suspension of strain cells was introduced into agar wells (in the amount of 100 µl per well), made in a dense nutrient medium with a sterile cork drill with a diameter of 11 mm. The volume of the nutrient medium in each Petri dish was 20 ml. As a control, the mold fungus *Aspergillus niger* VKM F-1754 was cultured without KE1.

The mold Aspergillus niger VKM F-1754 was passivated into the center of the Petri dish by the imprint method [11]. Crops were incubated at 25°C for 4 days. The mushroom colony diameter was measured every 24 hours and the averaged values were processed as previously described [12, 13].

The reliability of the obtained data was evaluated statistically using the difference analysis method [14].

3 Results and Discussion

Cultural and morphological properties. Colony formation by the KE1 strain on 3% peptone agar was observed on the second day of incubation at 35°C. The colonies were white with luster, opaque, broad, convex, the edge was not even. During the week the edges of the colonies were smoothed.

The cells of the KE1 strain are rod-shaped, arranged singly or in pairs.

The isolated isolate showed proteolytic, cellulolytic, lipolytic, amylolytic, catalase activity. Capable of releasing ammonia. The test for the presence of nitrate reductase was positive, which indicates the ability of the culture to nitrogen fixation and denitrification. At the same time, the strain did not show lecithinase, urease activity, does not form indole and hydrogen sulfide. Proteases are widely used in the food and dairy industries. Alkaline proteases have great potential for use in the washing and leather industry due to the growing trend towards the development of environmentally friendly technologies [15].

Hydrolytic enzymes, such as cellulases/xylanases, account for a significant share of the global enzyme market and are thus of great importance from an industrial and biotechnological point of view. The main factors driving the global hydrolytic enzyme market are a wide range of effective applications, niche products, environmental friendliness and reduced use and exploitation of chemicals [16].

Amylases represent a particularly important group of industrial enzymes, responsible for approximately 25% of the world's enzyme market. They present clear advantages: rapid growth of microorganisms and their great biochemical diversity, which leads to a wide variety of catalytic activities; regular availability owing to the absence of seasonal fluctuations, and ease of genetic manipulation, which offers the possibility of increased production and excretion of enzymes, as well as modified performance characteristics [17].

Catalases represent a class of enzymes which has found its place among industrially relevant biocatalysts due to their exceptional catalytic rate and high stability. Textile bleaching prior to the dyeing process is the main application and has been performed on a large scale for the past few decades [18].

KE1 isolate grows well on sucrose, D-glucose, lactose, D-fructose, sodium citrate, sodium acetate and does not grow on salicylic, sorbic, benzoic, tartaric acids, lead acetate, potassium-sodium tartrate.

In relation to oxygen, the culture turned out to be aerobic.

The KE1 isolate turned out to be similar in its features to the description for representatives of the genus Bacillus [19], namely, the Gram reaction, the ability to form spores, the ratio to oxygen and metabolic activity.

As a result of the alignment of the 16S rRNA gene sequence, a final sequence of 1,489 bp was obtained, which was used in further analysis. The sequence is stored in the GenBank database under the number... After analysis in NCBI BLAST, there is a complete similarity of the target strain with Bacillus subtilis. At the same time, it was not possible to identify a specific strain due to intraspecific monomorphism of 16S rRNA in Bacillus subtilis.

There is evidence that many representatives of this bacterium are biotechnologically significant.

Thus, there are known producers of metabolites that have a positive effect on plants due to the production of phytohormones, dissolution of inorganic phosphates, synthesis of organic acids, antagonism to phytopathogenic fungi, etc. [20,21] B. subtilis bacteria are widely distributed in the environment, since many wild strains are able to form biofilms on the surface of plant roots [22,23]. This type of microorganisms has suppressive qualities in vitro in relation to more than 20 types of phytopathogenic organisms due to the ability to produce a significant number of secondary metabolites: cyclic lipopeptides, polypeptides,

proteins and non-peptide compounds [24,25]. These substances, mainly peptides, have either ribosomal or non-ribosomal origin [26].

According to the literature data, the phospholipid antibiotic bacilizocin is produced inside B. subtilis 168 cells immediately after the cessation of growth and before the formation of thermostable spores. Its activity is more pronounced against the eukaryotic organism Sacharomyces cerevisiae, as well as the lower fungi Candida pseudotropicalis and Cryptococus neoformans, which are characterized by non-thread-like growth [27,28]. In particular, most strains of B. subtilis exhibit antagonistic activity in relation to pathogens of root rot of cereals of the genus Pseudomonas [29,30].

In this way, Bacillus sp. produces a significant number of biologically active metabolites having a diverse chemical structure: cyclic lipopeptides, proteins, polypeptides, ketones, polyene compounds and a number of others. The ability of bacteria to synthesize compounds of a certain structure implies the presence of a specific mechanism of action on a phytopathogenic object, and also explains the biological activity of a certain strain against specific microorganisms.

During the evaluation of the specific growth of the culture at different temperature values, it was revealed that the highest value was at 35°C. However, it was revealed that at 30°C the specific increase was lower than at 25°C. This may be due to the formation of a film on the interface of the phases. Large conglomerates could distort the optical density.

It was revealed that KE1 grows well on a medium containing 3% peptone for 20 hours, since the specific increase exceeded by 96% the variant cultivated in a mineral medium without an organic substrate. When Na-carboxymethylcellulose was added, the increase decreased by 86%, and with starch – 87% relative to 3% peptone.

When cultivating KE1 in the presence of 1% starch and Na-CMC for 6 days, it was revealed that the specific increase in the variant with carboxymethyl cellulose exceeded the growth with starch by 40%.

During the evaluation of the antagonistic activity of the KE1 strain, it was revealed that the specific increase (R) of the control variants was the same, however, for Ralstonia sp. the increase in the presence of KE1 decreased by 11%, while the presence of Ralstonia sp had no inhibitory or stimulating effect on the isolated strain.

The change in the number of isolate KE1 and Ralstonia sp. within 3 days, it did not show a significant difference. This may be due to the active film formation of KE1, which may affect the number of colony-forming units, as large conglomerates are formed.

When cultivating the strains together, it was found that during two days of incubation, the difference in the logarithm of the number of embryos in the culture fluid was insignificant. On the third day, the number of units of the KE1 isolate decreased by 25% relative to Ralstonia sp. Nevertheless, on the 4th day of incubation, it was revealed that the logarithm of the number of KE1 exceeded the number of ralstonia colonies by 3%. At the same time, the degree of inhibition (IR) of ralstonia during co-cultivation for 3 days was 17% relative to the control - the change in the number of ralstonia in pure culture.

Based on the parameter of the degree of inhibition for ralstonia relative to the control group, it was found that on the first day of incubation, this indicator was 10%, whereas on the 2nd - only 0.3%. Only on day 3, the parameter value was 17%. Based on this, it can be assumed that the isolate reduces the growth activity of ralstonia on day 3, however, the presence of KE1 has an inhibitory effect as the population develops in comparison with the control. Only on day 2, this effect is minimal, which may be due to the adaptation of cultures to the accumulation of metabolites or depletion of the substrate.

This effect is also manifested in the degree of inhibition of KE1 relative to the pure culture of the isolate. Despite the fact that the native strain exerts inhibitory activity on day 3, it itself was characterized by a change in its number and the IR parameter was 14%.

During the evaluation of the antagonistic potential of the isolated strain KE1 against E. coli O157:H7 by the method of agar disks, zones of suppression of the growth of E. coli culture were identified.

When cultivating strains together in a liquid nutrient medium (3% peptone), it was found that the logarithm of the number of colony-forming units decreased by 4.4% on the first day, whereas in the control variant the number increased by 18%. On the second day, it was shown that relative to the initial number, in the presence of KE1, the number of E. coli O157:H7 embryos decreased by 11.3%, whereas for the control group it increased by 47%. During subsequent incubation, the number of crops didn't change.

On the third day, the number of test culture units of the control variant decreased, relative to the second day, by 15%, whereas in the presence of KE1, an increase in the number of E. coli was observed by 26%. Thus, the rank of inhibition (IR) on the second day was higher than on the second and amounted to 29%. However, no inhibition was observed during further cultivation.

The presence of E. coli during KE1 incubation had an inhibitory effect on the second day, where the logarithm of the number of colony-forming units of the isolate decreased by 9% relative to the previous day. At the same time, in the control variant, there was an increase in the number by 6%. However, on the third day of incubation, the number of colonies increased in the presence of E. coli O157:H7 by 30%, whereas without E. coli - by only 2%.

The rank of inhibition (IR) of the KE1 culture on the second day was 15%. Thus, it can be assumed that the isolated strain has a bacteriostatic property in relation to E. coli.

During the evaluation of antifungal activity, it was revealed that in the presence of the KE1 strain, the average square diameter of the mold fungus did not change on the second day of cultivation, whereas in the control variant it differed by 45%. Further measurements showed that the diameter of A. unguis VCM F-1754 changed insignificantly, whereas in the control variant, the colonization of the nutrient medium by mold fungus turned out to be more effective.

It was revealed that in the presence of the KE1 strain in the medium, the colony growth rate constant decreases by 88% relative to the variant without the culture under study. At the same time, the rank of inhibition of A. unguis VCM F-1754 was 51%.

Thus, there is a possible interaction of the isolate with such test cultures as Ralstonia sp., E. coli O157:H7 and A. unguis VKM F-1754, where the KE1 strain showed bacterio- and mycostatic properties. The effects may be due to the formation of substances that have antibiotic potential. There is information in the literature that many representatives of the genus Bacillus are capable of biosynthesis of such substances. In addition to the inhibitory activity on the number, the influence of microorganisms may be associated with the impact on the signaling or virulent systems of a competitor, which leads to a change in the main pathway of metabolism.

4 Conclusions

During developing new environmentally friendly preparations to protect plants from phytopathogens, it is necessary to take into account the cultural characteristics and antagonistic properties of microorganisms. We have studied the morphological and cultural characteristics of the aboriginal strain KE1 isolated from the soils of the Belgorod region. Studies of the microorganism using an atomic force microscope showed that the cells and the KE1 strain are rod-shaped, arranged singly or in pairs. The conditions of cultivation of the bacterium were revealed, which showed rather unpretentious conditions with the composition of the medium containing 3% peptone for 20 hours, the specific increase in biomass exceeded 96%, further work in this direction will allow choosing an economically

advantageous technology for the production of the microorganism. Also, it is worth noting that the KE1 strain suppresses the growth of cultures of Escherichia coli O157:H7, Aspergillus unguis VKM F-1754 and bacteria of the genus Ralstonia. Thus, the data obtained indicate that the isolated KE1 strain is a promising object for expanding the range of producing strains that can become the basis for the development of new technologies.

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