

THE PROPERTIES OF BACTERIOCINS OBTAINED UNDER THE DIFFERENT CONDITIONS

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Abstract: Bacteriocins are substances of protein nature, which are produced by many strains of microorganisms. Due to the fact that bacteriocins are effectively used as antimicrobial agents in medicine and food industry, isolation and study of the properties of new bacteriocins-producing strains is very important. It should be noted that production of bacteriocins by microbial cells directly depends on strain cultivation conditions and methods of bacteriocin isolation. Among other cultivation parameters such as temperature and the active acidity of the culture medium, the duration of cultivation directly influences the synthesis of bacteriocins. Thus, the selection of the optimum duration of strains cultivation and method of bacteriocin isolation would allow us to increase significantly the bacteriocin production and, hence the antimicrobial activity of the strains. Therefore, determination of the optimum duration of cultivation and method of bacteriocins isolation is a very important step for getting bacteriocins with maximum activity. In this study, the isolation of microbial cultures from the fresh onion surface and the identification of these strains by sequencing of the 16S RNA gene were carried out. Also their morphological and physiological-and-biochemical properties were studied. The optimum cultivation duration was performed by using the determination of the antimicrobial activity in different time intervals. The strain cultivation was carried out during 24 hours. Three methods were used to isolate bacteriocins. They were based on the using of activated carbon, ammonium sulfate, and chloroform. The antimicrobial activity of bacteriocins was studied by means of the disk diffusion method. The identification of the isolated strains has revealed that the strains belong to *Bacillus safensis* and *Bacillus pumilus* species. It was found that the bacteriocins had the greatest antimicrobial activity when being cultivated for 18 hours and using method based on ammonium sulfate precipitation.

Keywords: Bacteriocins, duration of cultivation, method of bacteriocin isolation, antimicrobial activity

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INTRODUCTION

Bacteria are able to synthesize metabolites being used in human life such as antibiotics, enzymes, amino acids and etc. [1, 2]. Thus, the isolation and study of the properties of new bacteriocin-producing strains are very important. Nowadays, special attention is paid to creating new antibiotics and antimicrobial agents due to wide spread of antibiotic resistant pathogenic strains [1, 3]. Some antimicrobial agents such as bacteriophages, probiotic bacteria [4], antimicrobial peptides [5, 6], and bacteriocins [7] have already been studied. They are produced by bacteria and are an alternative to antibiotics. Having a number of advantages, bacteriocins are one of the most promising components to create antibiotics; therefore, they are a subject of study nowadays.

Bacteriocins are anti-infection agents of protein nature produced by many bacterial strains which have a broad spectrum of antimicrobial activity and are safe for humans. Due to this fact, the use of bacteriocins in medical and food industry is future-oriented.

Bacteriocins are a large group of heterogeneous bacterial antagonists differing considerably from one another in the molecular mass, biochemical properties, and the mechanism of action.

Bacillus strains have the ability to synthesize a wide spectrum of bacteriocins. The main bacteriocins-producing strains are *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus circulans*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus amyloliquefaciens* [8, 9, 10].

Generally, *Bacillus* strains produce peptides and lipopeptide antibiotics [11] and have the ability to synthesize a wide spectrum of bacteriocins such as batislin and fungumitsin, plipastatin and surfaktin [12], koagulin [13], tochiin [14], amilolichin [15]. A broad spectrum of antimicrobial activity of bacteriocins being produced by strains of *Bacillus* allows them not only to inhibit the growth of Gram-positive microorganisms but also to inhibit Gram-negative bacteria, yeast and fungi, which are pathogenic for human and animals.

At present, a lot of information about bacteriocins has been studied: their genetic traits, mechanisms of action, molecular mass that allows us to classify bacteriocins, and strains being more sensitive to bacteriocins.

Mechanisms of bacteriocins actions are found to be different but generally bacteriocins exert their antimicrobial effect by breaking the cell wall or membrane of target organisms, either by inhibiting the biosynthesis of the cell wall or causing pore formation which leads to death [16].

Due to the ability of *Bacillus* strains to synthesize a large number of antimicrobial agents with a broad spectrum of the antimicrobial activity, the study of these strains is important for discovering new methods of their application [17]. Over the last five years new prospects for using bacteriocins have been offered, especially in pharmaceutical and food industry. Thus, the study of new bacteriocin types opens new promising advantages of their use.

Many researchers have concluded that bacteriocins have the ability to kill closely related species [18]. However, more detailed study showed that bacteriocins can take various forms and also exert the antimicrobial activity against other species. Practically, a range of antagonistic activity may be extended by regulating cultivation conditions of bacteriocins and by selecting the optimum isolation method. This would allow us to weaken cell barriers of target organisms and to increase the bacteriocin production [19, 20]. Therefore, the selection of cultivation duration of bacteriocins and method of their isolation is of great importance for producing bacteriocins with maximum antimicrobial activity [21].

In this study, we have given the results on the determination of the optimum cultivation duration of *Bacillus safensis* and *Bacillus pumilus* strains, and that of the optimum isolation method of bacteriocins which are produced by the strains.

OBJECTS AND METHODS OF STUDY

The subjects of research were *Bacillus safensis* B-12180 and *Bacillus pumilus* B-12182 from the Russian National Collection of Industrial Microorganisms (VKPM), having been isolated at the Kemerovo Institute of Food Science and Technology (University).

At various stages of the research the following materials were used: Bacto Peptone, beef extract, sodium chloride, yeast extract, dry bacterial agar, ammonium citrate, sodium acetate, disodium phosphate, magnesium sulfate heptahydrate, manganese sulfate pentahydrate, ethanol, activated carbon, ammonium sulfate, chloroform (Russia); glucose (Belarus); Tween, filters Millex-GV (USA); and antibiotics.

Isolation of microbial cultures. The cultures were isolated from the fresh onion surface. For this, a cut-up onion was put into a liquid culture medium and cultivated at 30 ± 2 , 37 ± 2 , and 45 ± 2 °C from 1 to 5 hours. After growing biomass, the cultivation was carried out onto a solid culture medium which contains (in g/l) NaCl 12.0, Bacto Peptone 6.0, yeast extract 6.0, and dry bacterial agar 10.0. In order to obtain pure

cultures, we performed inoculations every day until single colonies were formed. The identification of isolated colonies was carried out according to morphological properties, sporulation, and cell size by using Gram reaction method, oxidase, and catalase tests. For more accurate identification, a genetic analysis of the strains was carried out by sequencing of the 16S RNA gene [22].

Antibiotic resistance. Antimicrobial resistance of the isolated strains was studied by means of the disk diffusion method. The strains were cultivated on the solid culture medium containing (g/l) Bacto Peptone 10.0, beef extract 10.0, yeast extract 5.0, glucose 20.0, Tween 1.0, ammonium citrate 2.0, sodium acetate 5.0, disodium phosphate 2.0, magnesium sulfate heptahydrate 0.1, manganese sulfate pentahydrate 0.05, and dry bacterial agar 10.0. The samples were cultivated at the temperature of 30 ± 2 °C and pH of 6.5 for 24 hours.

Determination of the optimum duration of cultivation. The strains were cultivated on the liquid medium containing (g/l) Bacto Peptone 10.0, beef extract 10.0, yeast extract 5.0, glucose 20.0, Tween 1.0, ammonium citrate 2.0, sodium acetate 5.0, disodium phosphate 2.0, magnesium sulfate heptahydrate 0.1, and manganese sulfate pentahydrate 0.05. The samples were cultivated at 30 ± 2 °C for 24 hours (pH 6.5). The cultural liquid was subjected to centrifugation in order to separate the supernatant. Centrifugation was carried out at 7,000 g for 10 min. The supernatant was filtered using Millex-GV filters (0.22 µm). The antimicrobial activity against *Escherichia coli* B-6954 strain was determined.

Determination of the medium composition. Available sources of carbon and nitrogen were chosen. During experiments we varied sources of nitrogen (peptone, tryptone, yeast extract), carbon (glucose, fructose, sucrose), and chloride (sodium chloride, calcium chloride), and also the ratio added components expressed in percent. Composition of the culture media is given in Table 1. The selection of a suitable medium was carried out by measuring the concentration of recombinant protein in the medium every 2 hours for 4 – 20 hours.

Determination of antimicrobial activity. The antimicrobial activity of bacteriocins was studied by means of the disk diffusion method [23]. Beef-extract agar was poured into Petri dish, dried under UV lamp for 1 hour after which the test microorganisms were inoculated onto the medium surface and dried again for 30 minutes with opened foil. Further, sterilized disks with 10 µl of bacteriocins were put onto the medium surface, dried for 15 minutes, after which the dish was inverted, put into a thermostate for 15 minutes and inverted again. After aerobic incubation in the range from 30 ± 2 up to 37 ± 2 °C for 18 to 24 hours, the diameter of zones of inhabitation was measured.

The method of bacteriocin isolation based on using activated carbon. The strain cultivation was carried out in the liquid culture medium MRS. The cultural liquid was concentrated on hollow fibers then, sodium chloride was added and stirred in on a laboratory

rocker. The suspension then was centrifuged, pH of the supernatant was adjusted up to 3.0, and the obtained suspension was centrifuged. Water was added to the precipitate, after suspending the precipitate, ethanol was added, and the suspension was incubated at $0 \pm 2^\circ\text{C}$ for 30 minutes and centrifuged again. After removing ethanol, water and activated carbon were added into the solution and centrifuged in order to remove impurities adsorbed on carbon. Obtained aqueous solution was filtered through a membrane.

The method of bacteriocin isolation based on their precipitation with ammonium sulfate. The strain cultivation was carried out in the liquid culture medium MRS. The culture was centrifuged at 4,200 g for 30 minutes, after which ammonium sulfate was added up to 90 per cent saturation to sediment bacteriocins. After centrifugation at 4,200 g for 40 min, the precipitate was dissolved in 20 mM acetate buffer with pH 5.0, and centrifuged again at 4,200 g for 30 min to separate undissolved precipitate which was washed again in the buffer, and centrifuged. The obtained supernatant was used to determine the antimicrobial activity.

The method of bacteriocin isolation based on using chloroform. The strains were cultivated in the liquid culture medium MRS. Chloroform was added to the supernatant, the obtained suspension was stirred by using a magnetic shaker for 20 minutes and centrifuged at 10,400 g and $12 \pm 2^\circ\text{C}$ for 20 min. The top organic layer was carefully poured off and 5–10 ml of 0.1 M Tris-buffer with pH 7.0 was added for resuspending. The contents of the bottles (sediments, the surface layer, remainders of chloroform, and the cultural medium) and the mixture were mixed and centrifuged again at 12,100 g for 15 minutes. Then the precipitate was separated from the remaining chloroform and the medium.

RESULTS AND DISCUSSION

The strain isolation. Having obtained pure cultures, two strains were isolated and their morphological, physiological and biochemical properties were studied.

The first strain was Gram-positive, it had rod-shaped cells, flat elevation, filiform edge, rough surface, beige color, and dense, uniform texture.

The second strain was Gram-positive, rod-shaped, scallop-edged, convex, smooth, white-colored, with soft uniform texture. The research of the isolated strains resistance to antibiotics showed that the first strain was less resistant to erythromycin, levofloxacin, ampicillin, cefotaxime, and vancomycin (inhibition zones were more than 25 mm). The strain showed higher resistance against levomycetin, tetracycline, and nitrofurantoin (inhibition zones were up to 20 mm).

The second strain demonstrated less resistance to amoxicillin, erythromycin, levofloxacin, doxycyclin, cefotaxime, and benzylpenicillin (inhibition zones were more than 25 mm). The strain turned out more resistant against levomycetin, ceftriaxone, amikacin, rifampicin, oxacillin, and nitrofurantoin (inhibition zones were up to 20 mm).

Identification of isolated strains. For the first strain, the results of the 16S rRNA gene sequence are given below:

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CTAATACATGCAGTCGAGCGGACAGAAGGGAG
CTTGCTCCCGGATGTTAGCGGCGGACGGGTGA
GTAACACGTGGGTAACCTGCCTGTAAGACTGG
GATAACTCCGGGAAACCGGAGCTAATACCGGAT
AGTTCCTTGAACCGCATGGTTCAAGGATGAAA
GACGGTTTTCGGCTGTCACTTACAGATGGACCCG
CGGCGCATTAGCTAGTTGGTGGGGTAATGGCTC
ACCAAGGCGACGATGCGTAGCCGACCTGAGAG
GGTGATCGGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGACGAGTAGGGAAT
CTTCCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGTTTTCGGATCGTA
AAGCTCTGTTGTTAGGGAAGAACAAGTGCGAG
AGTAACTGCTCGCACCTTGACGGTACCTAACCA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA
TTATTGGGCGTAAAGGGCTCGCAGGCGTTTTCT
TAAGTCTGATGTGAAAGCCCCGGCTCAACCG
GGGAGGGTCATTGGAAACTGGGAAACTTGAGT
GCAGAAGAGGAGAGTGGAATTCACGTGTAGC
GGTCAAATGCGTAGAGATGTGGAGGAACACCA
GTGGCGAAGGCGACTCTCTGGTCTGTACTGAC
GCTGAGGAGCGAAAGCGTGGGGAGCGAACAG.
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The phylogenetic tree (Fig. 1) was constructed to determine homology of the strain.

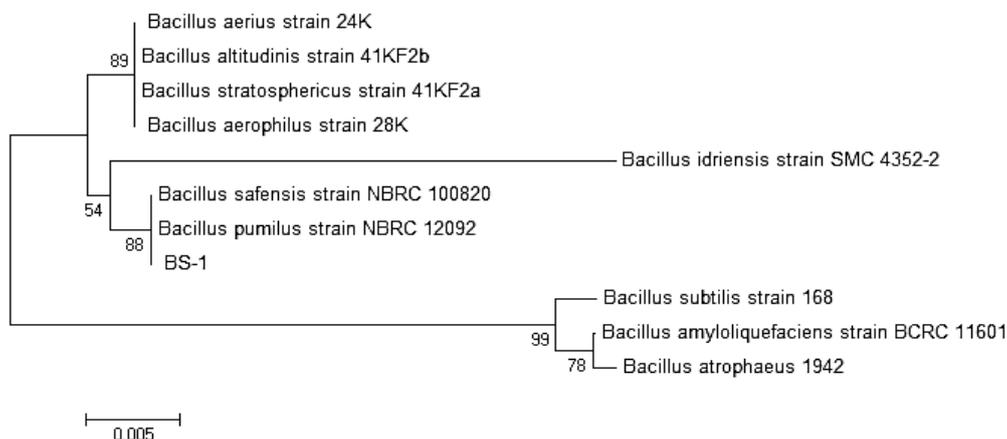


Fig. 1. Phylogenetic analysis of the first strain. BS-1 is the notation of the strain.

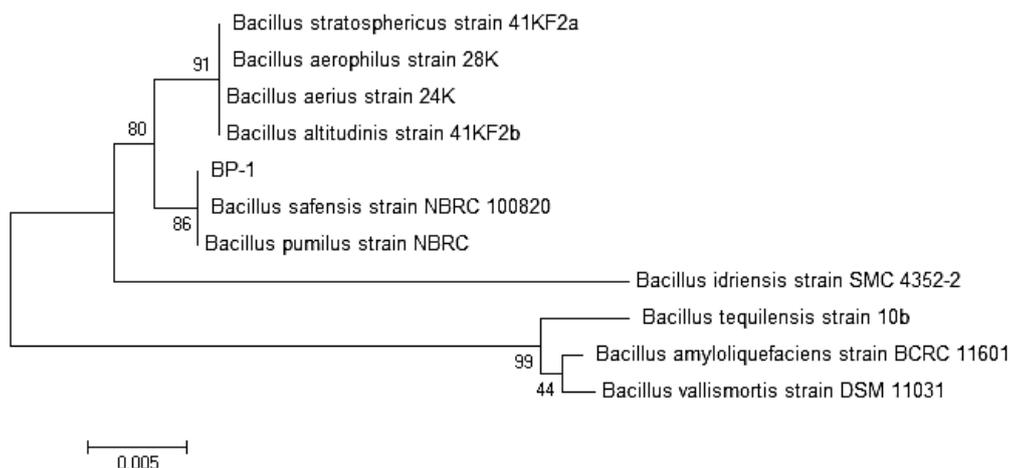


Fig. 2. The phylogenetic tree of the second strain. BP-1 is the notation of the strain.

Using nucleotide sequence of the isolated strain, its homology in relation to other strains of *Bacillus* was studied. The analysis was carried out by using NCBI data (national Center for Biotechnology Information). Ten nucleotide sequences with the highest homology to the studied strain were selected to build the phylogenetic tree for more accurate identification. The strains formed three main groups with the node stability 88, 89, and 99 per cent which indicates sufficiently high homology level of studied strains in formed groups. The strain of *Bacillus idriensis* SMC 4352-2 was isolated. From the results, the isolated strain was found to have the highest homology to *Bacillus safensis* and *Bacillus pumilus* strains. The analysis of morphological and physiological-and-biochemical properties revealed that the isolated strain belongs to *Bacillus safensis* specie.

For the first strain, the results of the 16S rRNA gene sequence are given below.

After the 16S rRNA gene sequencing, the following nucleotide sequence was obtained:

CTAATACATGCAGTCGAGCGGACAGAAGGGAGC
 TTGCTCCCGGATGTTAGCGGCGGACGGGTGAGT
 AACACGTGGGTAACCTGCCTGTAAGACTGGGAT
 AACTCCGGAAACCGGAGCTAATACCGGATAGT
 TCCTTGAACCGCATGGTTCAAGGATGAAAGACG
 GTTTCGGCTGTCACTTACAGATGGACCCGCGGC
 GCATTAGCTAGTTGGTGGGGTAATGGCTCACCA
 AGGCGACGATGCGTAGCCGACCTGAGAGGGTG
 ATCGGCCACACTGGGACTGAGACACGGCCAG
 ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
 GCAATGGACGAAAGTCTGACGGAGCAACGCCG
 CGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCT
 CTGTTGTTAGGGAAGAACAAGTGCAGAGAGTAA
 CTGCTCGCACCTTGACGGTACCTAACCAGAAAG
 CCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
 TACGTAGGTGGCAAGCGTTGTCCGGAATTATTG
 GGCGTAAAGGGCTCGCAGGCGGTTTTCTTAAAGTC
 TGATGTGAAAGCCCCGGCTCAACCGGGGAGG
 TCATTGGAAACTGGGAAACTTGAGTGCAGAAG
 AGGAGAGTGGAATCCACGTGTAGCGGTGAAAT

CGCTAGAGATGTGGAGGAACACCAGTGGCGAA
 GCGACTCTCTGGTCTGTACTGACGCTGAGGAG
 CGAAAGCGTGGGGAGCGAACAG.

Homology of the strain was determined by means of phylogenetic analysis. The results are in Figure 2.

The analysis of the 16S rRNA gene sequence of the isolated strain was performed to determine strains with the highest homology to the second isolated strain. In order to build the phylogenetic tree, strains with homology 97–99 per cent to the studied strain were chosen. The strains formed two main groups with the node stability 80 and 99 per cent. The homology level with bootstrap-value 70–100 per cent is considered to be high. That led to the conclusion that the homology level in the groups was high. The main groups were divided into three subgroups with the node stability 44–91 per cent. Strains of *Bacillus idriensis* SMC 4352-1 and *Bacillus tequilensis* BCRC 11601 were separated from the main groups. The phylogenetic analysis showed that strains of *Bacillus safensis* and *Bacillus pumilus* are closely related to the studied strain. Taking into account the morphological and physiological-and-biochemical properties, the studied strain was designated as *Bacillus pumilus*.

Determination of the optimum duration of cultivation. The optimum cultivation duration was determined by measuring the antimicrobial activity in different time intervals. The cultivation was carried out at $30 \pm 2^{\circ}\text{C}$ for 12, 18, and 24 hours (pH 6.5). The antimicrobial activity of bacteriocins against *Escherichia coli* B-6954 was studied by means of the disk diffusion method. The results are in Fig. 3 and Fig. 4.

It is seen from Fig. 3 that under the cultivation of *Bacillus safensis* strain for 12 hours, inhibition zones values varied from 7 up to 10 mm with a test culture. When cultivating for 18 hours, inhibition zones values were from 9 up to 11 mm, and for 24 hours the values were from 7 up to 10 mm. The strain showed the greatest activity against *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*.

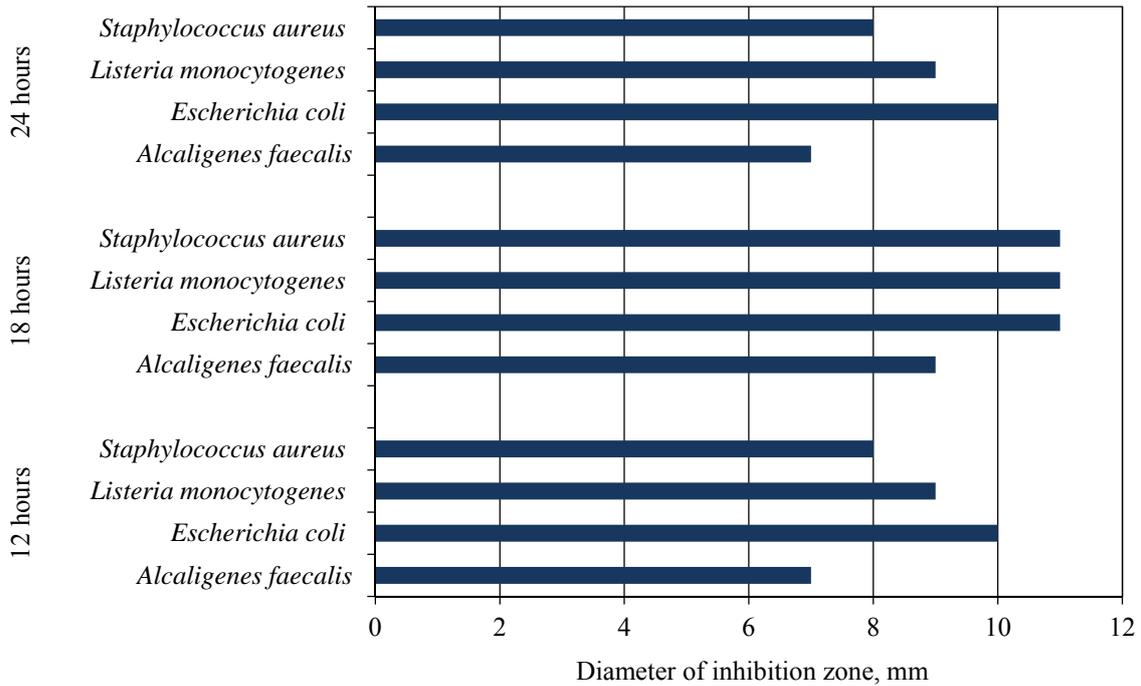


Fig. 3. The effect of the cultivation duration on the antimicrobial activity of *Bacillus safensis*.

Bacillus pumilus strain was cultivated for 12 hours and inhibition of pathogenic strains growth was observed. Inhibition zones were ranged from 7 up to 9 mm. With the increasing of time up to 18 hours, inhibition zones were ranged from 8 up to 10 mm. After cultivation of strain for 24 hours inhibition zones were from 7 up to 9 mm. It was found that *Bacillus pumilus* was more effective against *Staphylococcus aureus* strains.

Determination of the medium composition. Components of culture media and their concentrations

that were used for strain cultivation are represented in Table 1.

The bacteriocin synthesis intensity was determined by measuring the concentration of recombinant protein in the medium. The results are given in Fig. 5 and Fig. 6.

It is seen that *Bacillus safensis* and *Bacillus pumilus* strains had the minimum protein concentration on the culture medium No. 1, while the maximum concentration was observed on the medium No. 6.

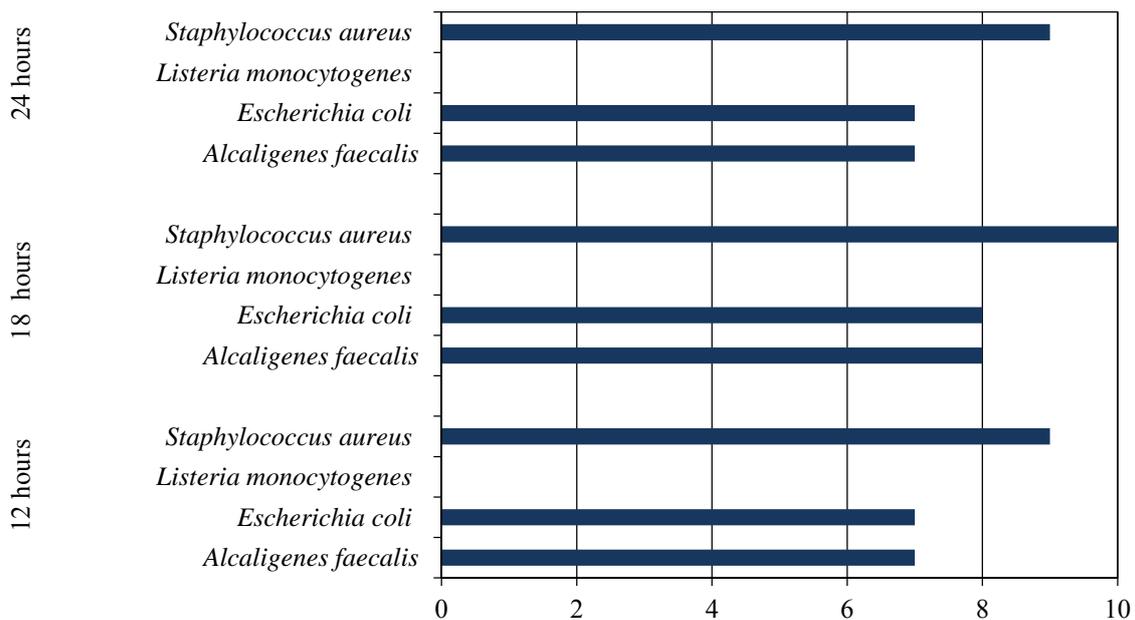


Fig. 4. The effect of the cultivation duration on the antimicrobial activity of *Bacillus pumilus*.

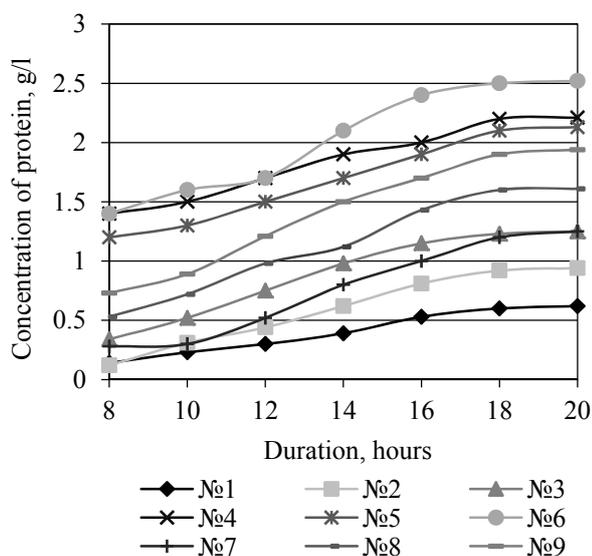
Table 1. Composition of culture media

Component	Mass content, g/dm ³								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
Peptone	12	12	12	–	–	–	–	–	–
Tryptone	–	–	–	12	12	12	–	–	–
Yeast extract	–	–	–	–	–	–	12	12	12
Pancreatic hydrolysate	12	12	12	–	–	–	12	12	12
Glucose	–	10	–	–	10	–	–	10	–
Fructose	10	–	–	10	–	–	10	–	–
Sucrose	–	–	12	–	–	12	–	–	12
Sodium acetate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Magnesium sulfate	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Ammonium sulfate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sodium chloride	6	6	5	5	5	6	6	6	5
Calcium chloride	2	2	3	3	3	2	2	2	3

Determination of the optimum method of bacteriocin isolation. Method of bacteriocin isolation considerably influences the activity and the number of isolated bacteriocins. Three methods of bacteriocin isolation were used. The first method is based on using activated carbon. The results are given in Table 2.

The results in Table 2 allow us to conclude that bacteriocins being produced by *Bacillus pumilus* strain had the inhibiting capacity against such pathogenic strains as *Leuconostoc mesenteroides*, *Escherichia coli*, *Enterobacter ludwigii*, *Salmonella enteric*, *Yersinia spp.*, *Staphylococcus aureus*, and *Alcaligenes faecalis*. The diameter of inhibition zones was 9–10 mm.

The *Bacillus safensis* strain showed higher antimicrobial activity to pathogenic strains than *Bacillus pumilus*. Inhibition zones were 14–16 mm in diameter.

**Fig. 5.** Bacteriocin production of *Bacillus safensis* strain in a culture medium, depending on its composition.

The second method of bacteriocin isolation was based on ammonium sulfate salting out with subsequent centrifugation and washing in the acetate buffer. The results of the antimicrobial activity of bacteriocins are represented in Table 3.

According to the results given in Table 3, the strain of *Bacillus safensis* was active against such strains as *Pseudomonas fluorescens*, *Candida albicans*, *Arthrobacter cummingsii*, *Alcaligenes faecalis*, *Escherichia coli*, *Enterobacter ludwigii*, *Erwinia aphidicola*, *Micrococcus luteus*, *Salmonella enterica*, *Listeria monocytogenes*, and *Staphylococcus aureus*. Inhibition zones were 14–18 mm.

The *Bacillus pumilus* strain has inhibited the growth of following test cultures: *Leuconostoc mesenteroides*, *Enterobacter ludwigii*, *Salmonella enterica*, *Yersinia spp.*, *Staphylococcus aureus*, *Alcaligenes faecalis*, and *Escherichia coli*. The inhibition zone diameters were 10–11 mm.

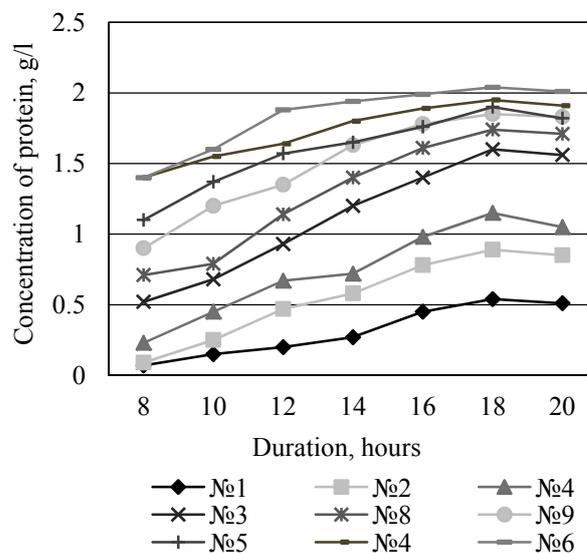
**Fig. 6.** Bacteriocin production of *Bacillus pumilus* strain in a culture medium, depending on its composition.

Table 2. Antimicrobial activity of bacteriocins isolated by using the first method

Test culture	Studied strain	
	Diameter of inhibition zones	
	<i>Bacillus safensis</i>	<i>Bacillus pumilus</i>
<i>Pseudomonas fluorescens</i>	14	0
<i>Pseudomonas aeruginosa</i>	0	0
<i>Candida albicans</i>	15	0
<i>Leuconostoc mesenteroides</i>	0	10
<i>Arthrobacter cummingsii</i>	14	0
<i>Alcaligenes faecalis</i>	15	8
<i>Escherichia coli</i>	16	9
<i>Enterobacter ludwigii</i>	15	9
<i>Erwinia aphidicola</i>	15	0
<i>Micrococcus luteus</i>	16	0
<i>Salmonella enterica</i>	16	9
<i>Listeria monocytogenes</i>	14	0
<i>Yersinia spp.</i>	0	10
<i>Staphylococcus aureus</i>	15	10

Table 3. Antimicrobial activity of bacteriocins isolated by using the second method

Test culture	Studied strain	
	Diameter of inhibition zones	
	<i>Bacillus safensis</i>	<i>Bacillus pumilus</i>
<i>Pseudomonas fluorescens</i>	14	0
<i>Pseudomonas aeruginosa</i>	0	0
<i>Candida albicans</i>	17	0
<i>Leuconostoc mesenteroides</i>	0	10
<i>Arthrobacter cummingsii</i>	15	0
<i>Alcaligenes faecalis</i>	16	9
<i>Escherichia coli</i>	18	10
<i>Enterobacter ludwigii</i>	18	11
<i>Erwinia aphidicola</i>	16	0
<i>Micrococcus luteus</i>	18	0
<i>Salmonella enterica</i>	18	10
<i>Listeria monocytogenes</i>	16	0
<i>Yersinia spp.</i>	0	11
<i>Staphylococcus aureus</i>	15	10

Table 4. Antimicrobial activity of bacteriocins isolated by using the third method

Test culture	Studied strain	
	Diameter of inhibition zones	
	<i>Bacillus safensis</i>	<i>Bacillus pumilus</i>
<i>Pseudomonas fluorescens</i>	14	0
<i>Pseudomonas aeruginosa</i>	0	0
<i>Candida albicans</i>	15	0
<i>Leuconostoc mesenteroides</i>	0	9
<i>Arthrobacter cummingsii</i>	13	0
<i>Alcaligenes faecalis</i>	0	8
<i>Escherichia coli</i>	15	8
<i>Enterobacter ludwigii</i>	15	0
<i>Erwinia aphidicola</i>	14	0
<i>Micrococcus luteus</i>	15	0
<i>Salmonella enterica</i>	15	8
<i>Listeria monocytogenes</i>	13	0
<i>Yersinia spp.</i>	0	0
<i>Staphylococcus aureus</i>	14	9

The third method allowed us to isolate bacteriocins by adding chloroform, isolating bacteriocins, centrifuging and resuspending in the Tris-buffer. The results are represented in Table 4.

It is seen from Table 4 that the *Bacillus safensis* strain has inhibited the growth of most pathogenic strains such as *Pseudomonas fluorescens*, *Candida albicans*, *Arthrobacter cummingsii*, *Escherichia coli*, *Enterobacter ludwigii*, *Erwinia aphidicola*, *Micrococcus luteus*, *Salmonella enterica*, *Listeria monocytogene*, and *Staphylococcus aureus*. The inhibition zones varied between 13 and 15 mm.

The *Bacillus pumilus* strain showed the antimicrobial activity to such pathogenic strains as *Listeria monocytogene*, *Salmonella enterica*, *Staphylococcus aureus*, *Alcaligenes faecalis*, and *Escherichia coli*. The inhibition zone diameters were 8–9 mm.

DISCUSSION

In this study, the isolation of two microbial cultures from the fresh onion surface was carried out and their morphological, genetic, physiological and biochemical properties were studied. The identification of the isolated strains has revealed that the strains belong to the species *Bacillus safensis* and *Bacillus pumilus*.

The optimum cultivation duration of *Bacillus safensis* and *Bacillus pumilus* strains was determined. The antimicrobial activity was found to be maximal after cultivation for 18 hours.

As the less antimicrobial activity was observed after cultivation duration of strains for 12 and 24 hours, 18 hours was considered as the optimum duration of cultivation.

It was found that the optimum culture medium for studied strains was the medium No. 6 where the maximum protein production was observed. The medium contained (in g/dm³) tryptone 12.0, sucrose 12.0, sodium acetate 0.5, magnesium sulfate 0.2, ammonium sulfate 0.5, sodium chloride 6.0, and calcium chloride 5.0.

It was revealed that the bacteriocins produced by *Bacillus safensis* and *Bacillus pumilus* strains had the

greatest antimicrobial activity when they were isolated using the method based on ammonium sulfate precipitation. This method was used for further work with bacteriocins.

The bacteriocins produced by *Bacillus pumilus* showed the antagonistic activity only against several pathogenic microorganisms, while the bacteriocins produced by *Bacillus safensis* were highly effective no matter which method of bacteriocin isolation was used.

Thus, the *Bacillus safensis* strain has been selected for further research, as the bacteriocins of this strain have high antagonistic activity and are one of the most promising components antibiotics creation.

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