

DETERMINATION OF THE INTENSITY OF BACTERIOCIN PRODUCTION BY STRAINS OF LACTIC ACID BACTERIA AND THEIR EFFECTIVENESS

M. I. Zimina^{a,*}, A. F. Gazieva^a, J. Pozo-Dengra^b, S. Yu. Noskova^a,
and A. Yu. Prosekov^c

^a Kemerovo Institute of Food Science and Technology (University),
Stroiteley blvd. 47, Kemerovo, 650056, Russian Federation

^b Biorizon Biotech, Science and Technology Park of Almería (PITA),
C/ Albert Einstein 15, 04131 Almería, Spain

^c Kemerovo State University,
Krasnaya Str. 6, Kemerovo, 650043, Russian Federation

* e-mail: mariia.zimina@list.ru

Received January 18, 2017; Accepted in revised form March 24, 2017; Published June 29, 2017

Abstract: The study of bacteriocins is one of the most relevant areas of research. This is due to the increase in the number of pathogenic microorganisms resistant to antibiotics. The bacteriocins of lactic acid bacteria have the most common application because of their safety in use. To assess the antimicrobial potential of bacteriocins of lactic acid bacteria and the possibilities for their further use, this study deals with the intensity of bacteriocin production by strains of lactic acid bacteria, the minimal inhibitory concentration of bacteriocins produced by these strains with respect to *E. coli* B-6954, as well as the antimicrobial activity of the produced bacteriocins with respect to some strains of pathogenic microorganisms. This work was carried out on the basis of the Kemerovo Technological Institute of Food Industry (Russia). The study objects were strains of microorganisms *Lactobacillus delbrueckii* B2455, *Lactobacillus paracasei* B2430, *Lactobacillus plantarum* B884. Biomass concentration was determined by spectrophotometric method. Study of antimicrobial activity in relation to a number of pathogenic strains was carried out in a culture fluid by optical density, and also by a disk-diffusion test. As a result, the maximum productivity value, characterizing the intensity of bacteriocin production, was observed for strain B884. However, the quality of bacteriocin (antimicrobial activity relative to the *E. coli* strain) with a lower productivity is higher for strain B2430. The minimum inhibitory concentration for the strains studied was: B2430 – $1.0 \cdot 10^6$ CFU/ml, B884 – $1.0 \cdot 10^8$ CFU/ml, B2455 – $1.0 \cdot 10^7$ CFU/ml. The best indicator of the minimum inhibitory concentration was observed for strain B2430. Thus the strain B2430 had the greatest antimicrobial activity, it had an inhibitory effect on 12 test cultures: *Escherichia coli*, *Alcaligenes faecalis*, *Candida albicans*, *Pseudomonas libanensis*, *Staphylococcus warneri*, *Erwinia aphidicola*, *Microbacterium foliorum*, *Bacillus licheniformis*, *Serratia plymuthica*, *Erwinia aphidicola*, *Bacillus endophyticus*, *Leuconostoc mesenteroides*. Thus, among the test *Lactobacillus* strains, the strain of *Lactobacillus paracasei* B2430 is promising for further study.

Keywords: Lactic acid bacteria, bacteriocins, antimicrobial activity, biomass concentration, minimum inhibitory concentration

DOI 10.21179/2308-4057-2017-1-108-117

Foods and Raw Materials, 2017, vol. 5, no. 1, pp. 108–117.

INTRODUCTION

Lactic acid bacteria (LAB) are a group of phylogenetically diverse gram-positive bacteria that have some common morphological characteristics, as well as metabolic and physiological features [1]. Lactic acid bacteria have GRAS safety status and are often used in the production of food products.

Lactic acid bacteria contain in human microbiota and have significant impact on human health [2, 3]. They produce a number of antimicrobial compounds, which include hydrogen peroxide, CO₂, diacetyl,

acetaldehyde, lactic acid, D-isomers of amino acids, bacteriocins [4].

There is increasing tendency in our society to use more natural and safety products such as biodegradable materials, biofuels and different products, obtained by microbial synthesis (enzymes, polysaccharides, amino acids, vitamins, organic acids, bacteriocins) [5].

Bacteriocins are a heterogeneous group of ribosomally synthesized peptides or proteins that have antimicrobial activity against other bacteria. Action mechanism of bacteriocins is based on creation of

pores in the membrane of targeted cells, which interferes with the membrane potential and kills the cells. Bacteriocins are normally synthesized by strains as a defense system, and they inhibit development of microorganisms related to the producing strain [6]. Currently, one of the most urgent areas of research is the development of new antimicrobial agents, which is associated with a wide spread of pathogenic strains that are resistant to antibiotics [7]. In connection with this, in recent years, the interest of scientists in the study of bacteriocins has been especially increased, this is due to the possibility of their use as bioconservants in food products in order to suppress development of pathogenic microflora [8, 9].

Bacteriocins are divided into separate classes. Lantibiotics of the first class include lantibiotics, which are small (< 10 kDa) thermally stable unmodified proteins. Second-class bacteriocins are divided into pediocin-like (subclass IIa) and bi-peptic bacteriocins (subclass IIb). The third class includes large (> 30 kDa) thermolabile proteins [10].

Due to the high antimicrobial potential of bacteriocins, their use is most common in medicine for the production of antimicrobial agents [11, 12]. Also bacteriocins may be used in the food industry for increasing the shelf life of products and providing food security [13, 14, 15]. The study of new types of bacteriocins is a topical issue in modern science, due to vast possibilities of their application, and in this connection this article is devoted to study of bacteriocins produced by lactic acid bacteria, the intensity of their production and the effectiveness of the action.

Thus, the study was intended to determine the intensity of bacteriocin production by the strains studied, as well as to determine the effectiveness of the action of bacteriocins against pathogenic strains to study the possibility of further use of bacteriocins.

OBJECTS AND METHODS OF STUDY

The following reagents were used as materials at different stages of the study: bacto-peptone, meat extract, citric acid ammonium (Component-Reactiv LLC, Russia); acetous sodium, sodium hydrogenphosphate (Belkhim LLC, Belarus); magnesium sulphate 7-water, manganese sulfate 5-water (Cation LLC, Russia); yeast extract, sodium chloride (Component-Reactiv LLC, Russia); Millex-GV filters (0.22 µm, Nihon "Millipore", USA), Müller-Hinton medium, Saburo medium (Lab-Biomed LLC, Russia).

Strains of microorganisms provided by VKPM FSUE "GosNIIgenetika" (<http://www.genetika.ru/vkpm>) were used as objects of study: *Lactobacillus delbrueckii* B2455, *Lactobacillus paracasei* B2430, *Lactobacillus plantarum* B884.

As test cultures, natural and medical strains of pathogenic microorganisms were used, which were designated as follows: No. 1 – *Bacillus mycoides* EMTC 9, No. 2 – *Salmonella enterica* ATCC 14028, No. 3 – *Micrococcus luteus* EMTC 1860, No. 4 – *Escherichia coli* ATCC 25922, No. 5 – *Bacillus cereus* EMTC 1949, No. 6 – *Alcaligenes faecalis* EMTC 1882, No. 7 –

Proteus vulgaris ATCC 63, No. 8 – *Pseudomonas fluorescens* EMTC 42, No. 9 – *Candida albicans* EMTC 34, No. 10 – *Pseudomonas aeruginosa* ATCC 9027, No. 11 – *Staphylococcus aureus* ATCC 25923, No. 12 – *Pseudomonas libanensis* EMTC 1853, No. 13 – *Staphylococcus warneri* EMTC 1854, No. 14 – *Erwinia aphidicola* EMTC 1857, No. 15 – *Microbacterium foliorum* EMTC 1858, No. 16 – *Bacillus licheniformis* EMTC 1859, No. 17 – *Serratia plymuthica* EMTC 1861, No. 18 – *Rahnella aquatilis* EMTC 1862, No. 19 – *Erwinia aphidicola* EMTC 1863, No. 20 – *Bacillus endophyticus* EMTC 1864, No. 21 – *Leuconostoc mesenteroides* EMTC 1865, No. 22 – *Enterococcus casseliflavus* EMTC 1866.

All strains were cultured in a fermenter (80% filling) in a liquid MRS medium at 37°C for 3 days. The obtained biomass was separated from the culture medium by centrifuging at 8000 g for 20 minutes. A culture fluid filtered through 0.22 µm membrane filters was used as the bacteriocin solution. The resulting sterile solution of metabolites was used for experiments.

A suspension of nocturnal broth cultures grown on standard culture media was taken for work. The number of microorganisms (titre) in the suspension was determined by the optical density (OD) at a wavelength of 595 nm.

Biomass concentration was determined with the UV-1800 spectrophotometer (Shimadzu, Japan) by measuring the light absorbance at 595 nm. The obtained result was recalculated on the dry biomass basis. The cellular biomass was measured on a dry weight basis. Cells were precipitated on Buprog (pre-boiled) filters with a pore size of 0.24 µm, washed, dried at 80°C and weighed.

The protein concentration was determined spectrophotometrically by measuring the light absorbance at 595 nm. The obtained result was recalculated on the basis of the protein weight according to the albumin calibration table.

Specific productivity, as the value of the product increase per unit time, was determined by the formulas:

$$Q_P(t_i) = \frac{P(t_i) - P(t_0)}{t_i - t_0}, \quad i = 1, 2, 3, \dots, \quad (1)$$

where $t_i \in (0, \infty)$, t_0 is a time at which the lag phase of the periodic cultivation process ends and exponential phase begins; it is obvious that $Q_P(0) = 0$.

The suspension of overnight broth cultures of test strains grown on a standard culture media for 24 hours at a temperature of 37 °C were used for the study. Cells from the agar surface were gathered with an inoculation loop and resuspended in NaCl solution to 0.5 units according to the McFarland standard. The test strains were grown in MRS broth for 24 hours at 37°C, then the culture fluid was centrifuged at 2000 rpm for 10 minutes and the supernatant fluid was separated. To separate cells, the supernatant fluid was filtered through Millex-GV filters. The culture fluid turbidity was adjusted to a value of 0.5 according to the McFarland standard (containing approximately

1.5×10^8 CFU/ml). Suspension optical density was adjusted spectrophotometrically: light absorbance was within the same range as 0.5 in the McFarland units (at OD 450 nm, optical density was within 0.08–0.13). Then, the obtained microbial suspension was subjected to a series of 1:10 dilutions, thus reaching a metabolite concentration from 10^8 to 10^1 CFU/ml. 180 μ l of the supernatant fluid of each test strain were added to the plate, then 20 μ l of test culture strains were added to each of them. The plate was incubated at 37°C for 24 hours. 200 μ l of sterile MRS broth and 180 μ l of pure medium with 20 μ l of each pathogen solution were used as controls. Bacterial growth was monitored by measuring the optical density during the culture process.

The culture media were prepared as follows: MRS medium, g/l: Bacto-peptone – 10.0; meat extract – 10.0; yeast extract – 5.0; glucose – 20.0; tween – 1.0; ammonium citrate – 2.0; sodium acetate – 5.0; sodium hydrogenphosphate – 2.0; magnesium sulphate 7-water – 0.1; manganese sulphate 5-water – 0.05. MRSA, dense medium, g/l: bacto-peptone – 10.0; meat extract – 10.0; yeast extract – 5.0; glucose – 20.0; tween – 1.0; ammonium citrate – 2.0; sodium acetate – 5.0; sodium hydrogenphosphate – 2.0; magnesium sulphate 7-water – 0.1; manganese sulfate 5-water – 0.05; agar – 20.0.

Antimicrobial activity of strains of lactic acid bacteria was additionally determined by means of agar-diffuse discs [16]. The test strain was plated on an agarized culture medium (MRSA) in the form of lawn and the lawn was covered with paper discs impregnated with lactic acid bacteria metabolites (10 μ l/disc). Disc with MRS medium was used as a control, a disc with a ciprofloxacin antibiotic (from a standard kit) was used as a reference substance. The plates were incubated at 37°C for 24 hours. The results were taken into account by the presence and size (in mm) of the transparent zone of non-growth of microorganisms around the disc.

The minimum inhibitory concentration was determined with respect to the isolated strains of bacteria by the microtitration technique. The

antimicrobial activity of the bacteriocins produced was evaluated relative to the strain of *E. coli*. A suspension of the *E. coli* strain without addition of bacteriocins was used as a control.

RESULTS AND DISCUSSION

The biomass concentration of strains of lactic acid bacteria and the activity of bacteriocins produced by them were determined by culturing in a MRS culture fluid, the study was carried out for 70 hours. The biomass concentration was determined spectrophotometrically. The antimicrobial activity of bacteriocins was determined by measuring inhibition zones, the study was carried out by a disc-diffusion test. The results obtained in the study of the dependence of concentration of bacteriocins and their activity on the culturing period are presented in Fig. 1–5.

The results obtained allowed to plot a chart showing the intensity of bacteriocins production during culturing of three test strains of lactic acid bacteria, as well as to show the intensity of use of bacteriocins from the culturing period for all three strains. Results are presented in Fig. 4–5.

When analyzing the results presented in Fig. 1–5, we concluded that the period of culturing at which the maximum bacteriocin concentration is observed and the duration at which their maximum antimicrobial activity is observed are different. Thus, the maximum concentration of bacteriocins in the culturing of strain B884 was observed with a culturing duration of 54 hours, while the maximum antimicrobial activity of this strain was observed with a culturing period of 18 hours. In strain B2430, the maximum concentration of bacteriocins was found during culturing for 44 hours, and the maximum activity was observed during culturing for 20 hours. For strain B2455, these values were 57 and 54 hours, respectively. To estimate the intensity of bacteriocin production by microbial strains, the productivity by the target product was calculated, which is defined as the magnitude of the product increase per unit time. The obtained results are represented in Table 1.

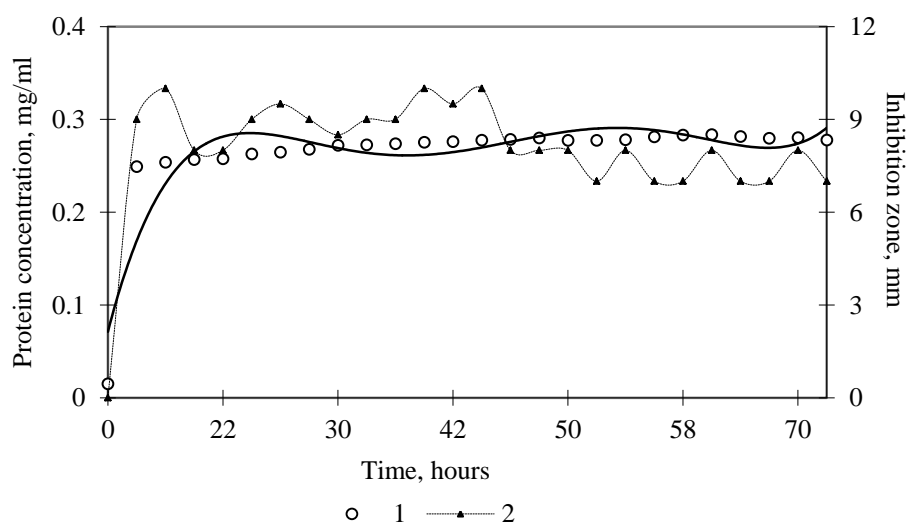


Fig. 1. Dependence of B884 bacteriocins concentration and their activity on the culturing period: (1) protein concentration, (2) activity.

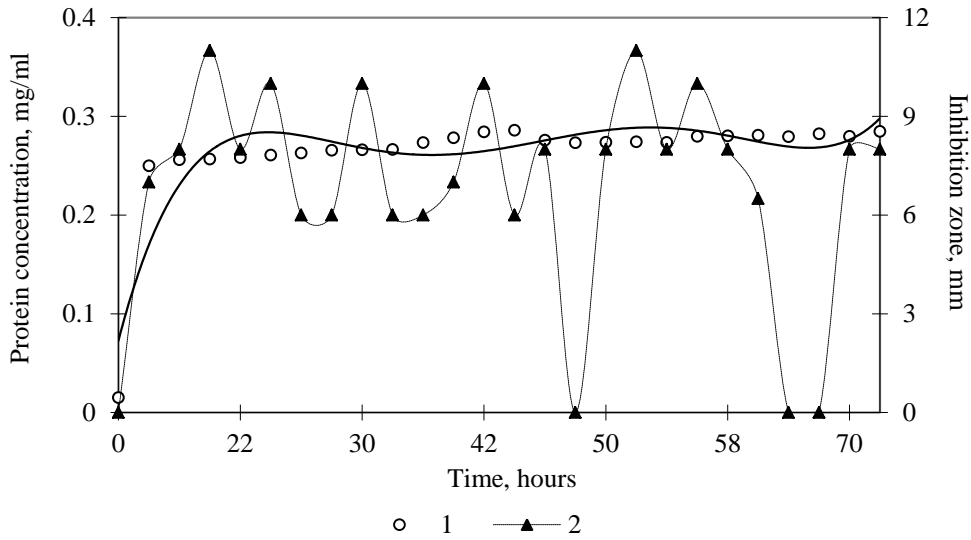


Fig. 2. Dependence of B2430 bacteriocins concentration and their activity on the culturing period: (1) protein concentration, (2) activity.

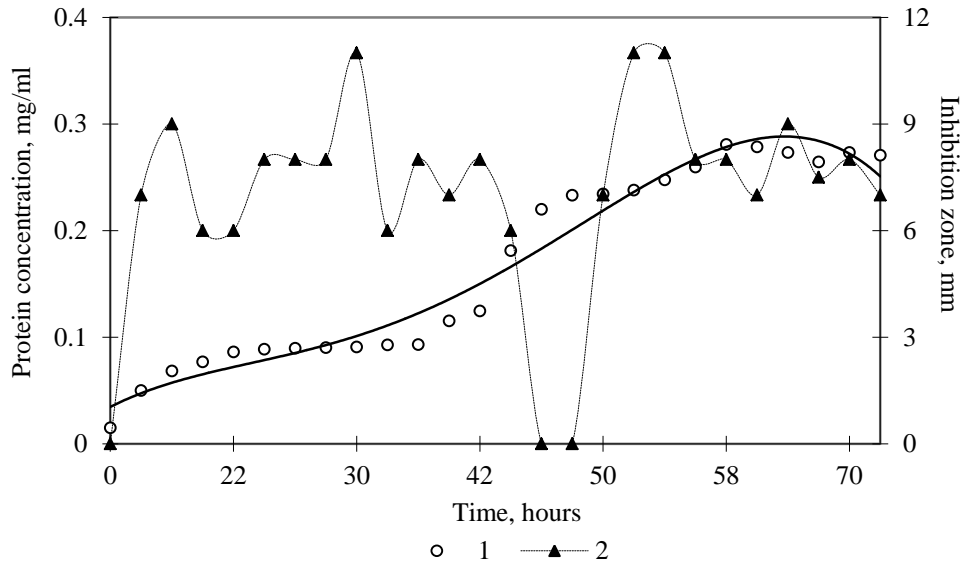


Fig. 3. Dependence of B2455 bacteriocins concentration and their activity on the culturing period: (1) protein concentration, (2) activity.

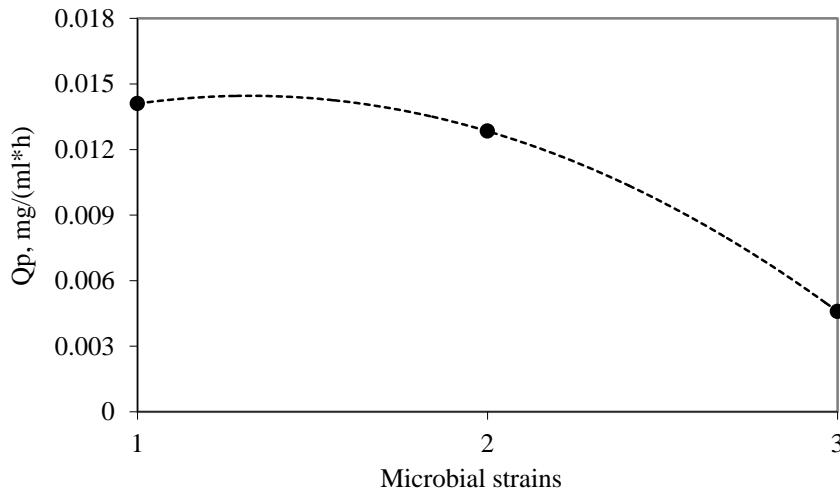


Fig. 4. Maximum intensity of bacteriocin production in the culturing of lactic acid bacteria: (1) B884, (2) B2430, (3) B2455.

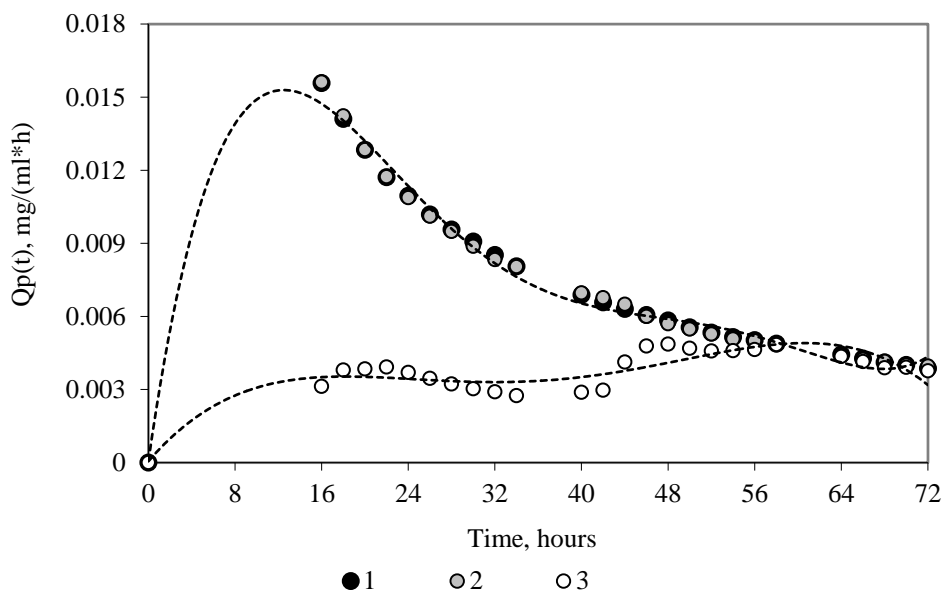


Fig. 5. Dependence of the intensity of bacteriocin production on the lactic acid bacteria culturing period: (1) B884, (2) B2430, (3) B2455.

Table 1. Intensity of bacteriocin production in the course of culturing strains

Strains	Inhibition zone, mm	Culturing period	Specific productivity
B884	10	18	0.0141
B2430	11	20	0.0128
B2455	11	54	0.0046

When analyzing the data given in Table 1, we came to the conclusion that the following productivities were observed for the strains studied: B884 – 0.0141, B2430 – 0.0128, B2455 – 0.0046. In the B884 strain, the inhibition zone of the test culture was 10 mm, in the strains B2430 and B2455 – 11 mm.

The minimal inhibitory concentration of isolated strains was determined. The study was carried out according to the microtitration technique. The antimicrobial activity of the bacteriocins produced was evaluated relative to the strain of *E. coli*. The results of the study are presented in Table 2 and in Figures 6–8.

When analyzing the results obtained in determining the minimum inhibitory concentration, the following conclusion was made. With a bacteriocin concentration of strain B884 of 10^8 CFU/ml, the growth of the *E. coli B-6954* test strain was significantly lower compared to the control. With a concentration of $1.0 \cdot 10^7$ CFU/ml, the growth of *E. coli B-6954* is lower than in the control, up to and including 4 hours of culturing. At the remaining

concentrations in the control sample, better indices were observed than in the samples with lactic acid bacteria. Thus, the minimum inhibitory concentration for strain B884 was $1.0 \cdot 10^8$ CFU/ml.

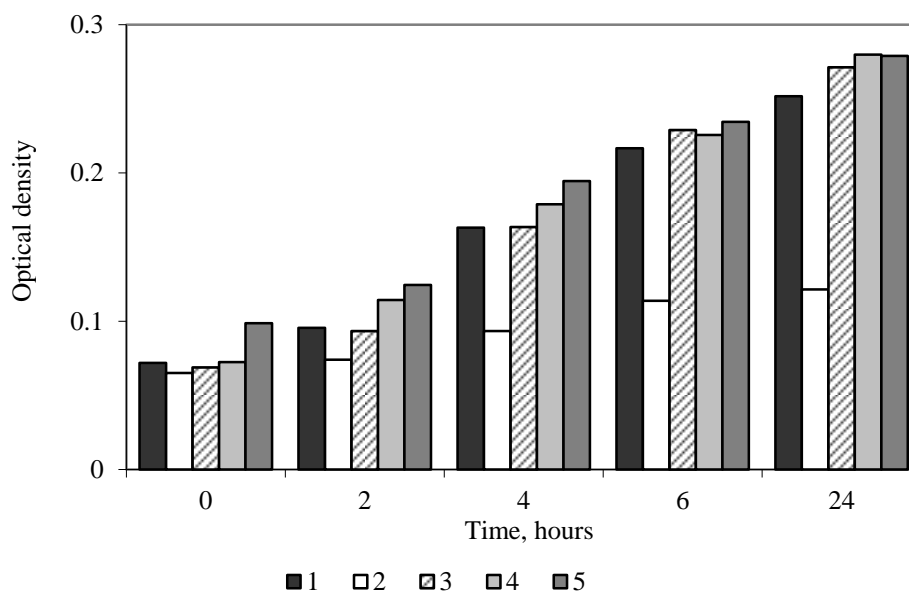
With bacteriocin concentrations of strain B2430 from $1.0 \cdot 10^8$ to $1.0 \cdot 10^6$ CFU/ml, a less intensive growth of the test culture was observed than in the control. With a concentration of $1.0 \cdot 10^5$ CFU/ml, the growth of *E. coli B-6954* is lower than in the control, up to and including 4 hours of culturing. The minimum concentration of strain B2430 was $1.0 \cdot 10^6$ CFU/ml.

When determining the minimum inhibitory concentration of strain B2455, it was found that at bacteriocin concentrations of $1.0 \cdot 10^8$ and $1.0 \cdot 10^7$ CFU/ml, the growth of the *E. coli B-6954* test strain was significantly lower compared to the control. At a concentration of $1.0 \cdot 10^6$ CFU/ml, the growth of *E. coli B-6954* was lower than in the control, up to 6 hours of culturing inclusive, but with a culture time of 24 hours, the *E. coli B-6954* concentration was higher than the control. The minimum inhibitory concentration of strain B2455 with respect to *E. coli B-6954* was $1.0 \cdot 10^7$ CFU/ml.

A study was made of the antimicrobial activity of bacteriocins produced by the strains under investigation, with respect to twenty-two pathogenic strains. The study was carried out by determining the optical density in a MRS culture fluid. The control sample was the culture of a pathogenic strain without the addition of bacteriocins. Results are presented in Fig. 9–11.

Table 2. Results of determination of the minimum inhibitory concentration of strains with respect to the strain *E. coli* B-6954

Culturing period, hrs	Optical density of the mixture at different concentrations of lactic acid bacteria, CFU/ml				
	$1.0 \cdot 10^8$	$1.0 \cdot 10^7$	$1.0 \cdot 10^6$	$1.0 \cdot 10^5$	control
B884					
0	0.0651	0.0689	0.0725	0.0987	0.0719
2	0.0741	0.0934	0.1143	0.1245	0.0955
4	0.0934	0.1629	0.1789	0.1945	0.1631
6	0.1138	0.2289	0.2256	0.2344	0.2166
24	0.1215	0.2713	0.2798	0.2789	0.2517
B2430					
0	0.0354	0.0389	0.0421	0.0600	0.0657
2	0.0400	0.0546	0.0778	0.0856	0.0923
4	0.0536	0.0765	0.0995	0.1110	0.1145
6	0.0876	0.1012	0.1245	0.1600	0.1521
24	0.1090	0.1233	0.1530	0.1855	0.1740
B2455					
0	0.0556	0.0643	0.0734	0.0880	0.0875
2	0.0720	0.0851	0.0965	0.1111	0.1098
4	0.0954	0.1011	0.1142	0.1344	0.1263
6	0.1160	0.1234	0.1536	0.1930	0.1752
24	0.1236	0.1355	0.1944	0.2194	0.1825

**Fig. 6.** Change in optical density at different concentrations of bacteriocins of the strain B884, in relation to the test culture *E. coli*: (1) control, (2) $1.0 \cdot 10^8$, (3) $1.0 \cdot 10^7$, (4) $1.0 \cdot 10^6$, (5) $1.0 \cdot 10^5$.

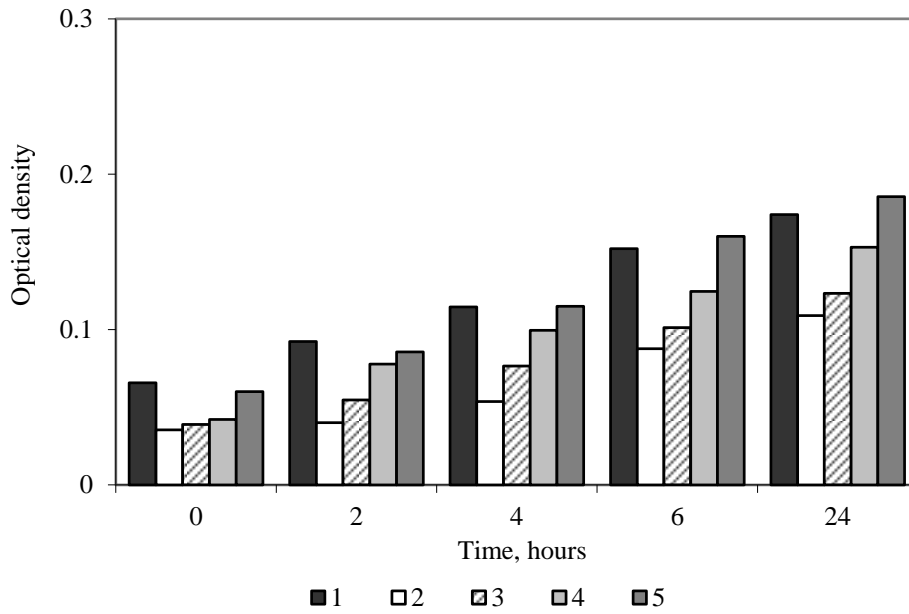


Fig. 7. Change in optical density at different concentrations of bacteriocins of the strain B2430, in relation to the test culture *E. coli*: (1) control, (2) $1.0 \cdot 10^8$, (3) $1.0 \cdot 10^7$, (4) $1.0 \cdot 10^6$, (5) $1.0 \cdot 10^5$.

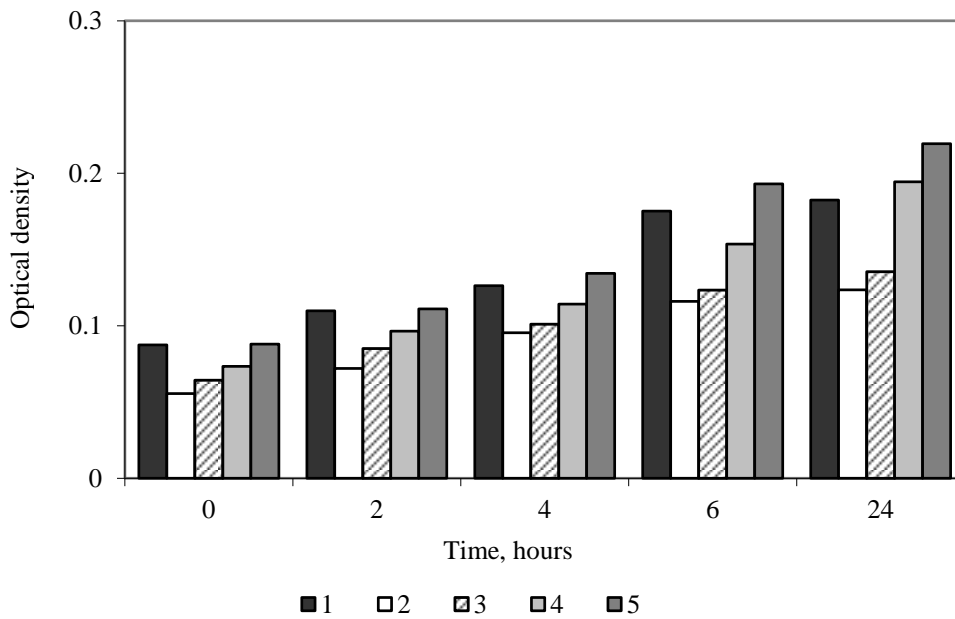


Fig. 8. Change in optical density at different concentrations of bacteriocins of the strain B2455, in relation to the test culture *E. coli*: (1) control, (2) $1.0 \cdot 10^8$, (3) $1.0 \cdot 10^7$, (4) $1.0 \cdot 10^6$, (5) $1.0 \cdot 10^5$.

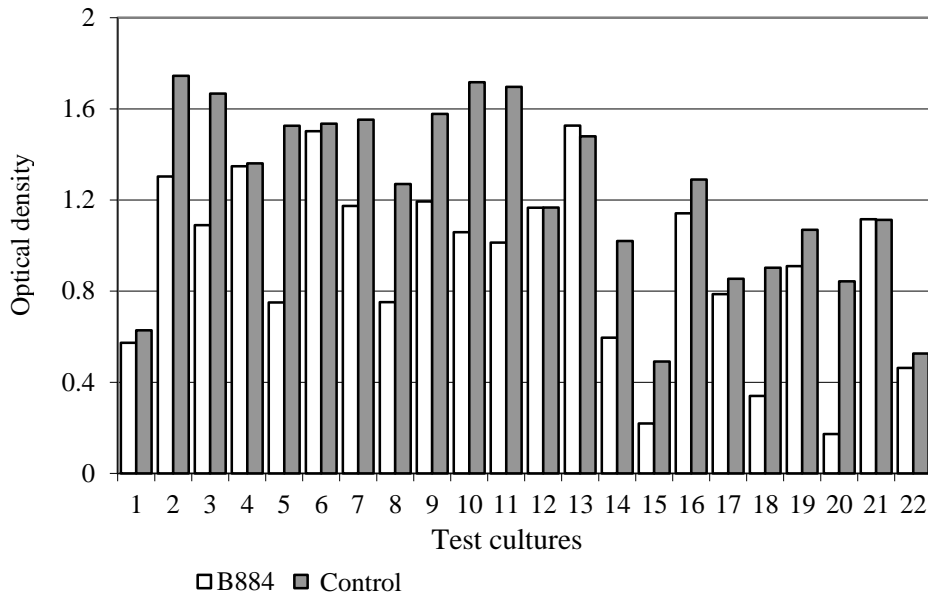


Fig. 9. Antimicrobial activity of strain B884: (1) *Bacillus mycoides*, (2) *Salmonella enterica*, (3) *Micrococcus luteus*, (4) *Escherichia coli*, (5) *Bacillus cereus*, (6) *Alcaligenes faecalis*, (7) *Proteus vulgaris*, (8) *Pseudomonas fluorescens*, (9) *Candida albicans*, (10) *Pseudomonas aeruginosa*, (11) *Staphylococcus aureus*, (12) *Pseudomonas libanensis*, (13) *Staphylococcus warneri*, (14) *Erwinia aphidicola*, (15) *Microbacterium foliorum*, (16) *Bacillus licheniformis*, (17) *Serratia plymuthica*, (18) *Rahnella aquatilis*, (19) *Erwinia aphidicola*, (20) *Bacillus endophyticus*, (21) *Leuconostoc mesenteroides*, (22) *Enterococcus casseliflavus*.

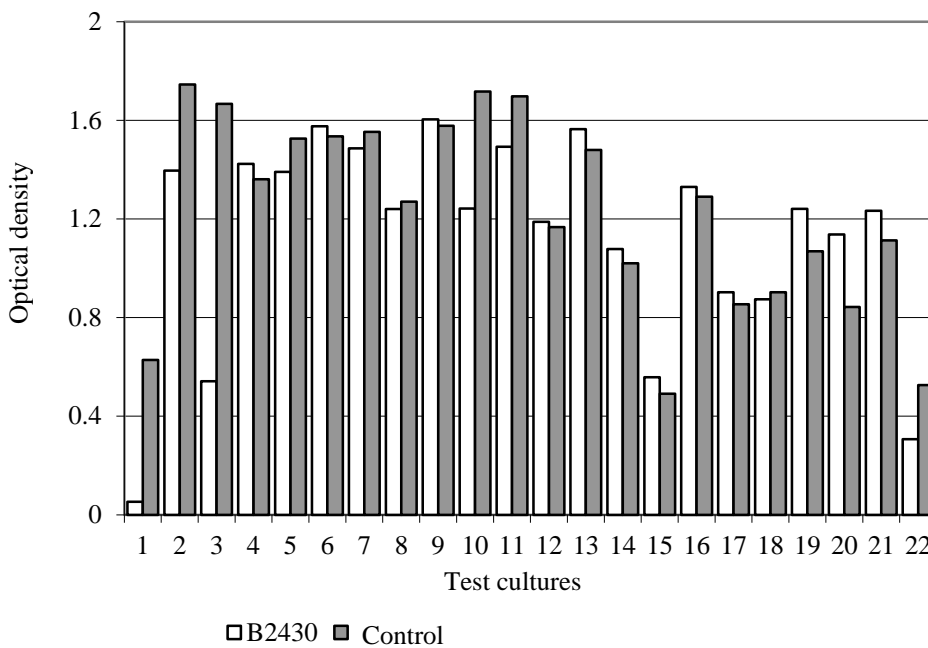


Fig. 10. Antimicrobial activity of strain B2430: (1) *Bacillus mycoides*, (2) *Salmonella enterica*, (3) *Micrococcus luteus*, (4) *Escherichia coli*, (5) *Bacillus cereus*, (6) *Alcaligenes faecalis*, (7) *Proteus vulgaris*, (8) *Pseudomonas fluorescens*, (9) *Candida albicans*, (10) *Pseudomonas aeruginosa*, (11) *Staphylococcus aureus*, (12) *Pseudomonas libanensis*, (13) *Staphylococcus warneri*, (14) *Erwinia aphidicola*, (15) *Microbacterium foliorum*, (16) *Bacillus licheniformis*, (17) *Serratia plymuthica*, (18) *Rahnella aquatilis*, (19) *Erwinia aphidicola*, (20) *Bacillus endophyticus*, (21) *Leuconostoc mesenteroides*, (22) *Enterococcus casseliflavus*.

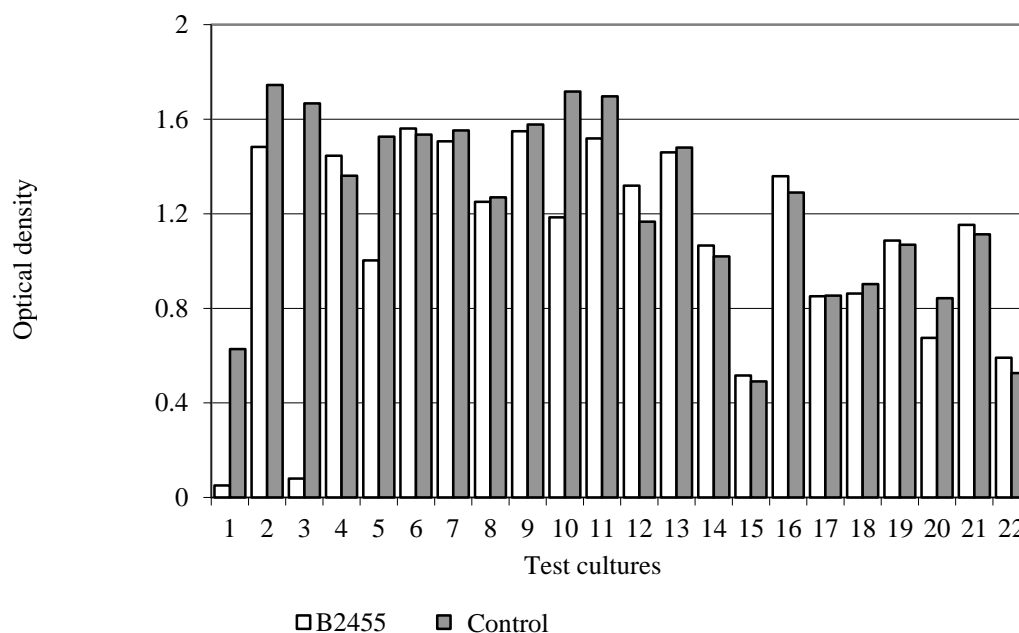


Fig. 11. Antimicrobial activity of strain B2455: (1) *Bacillus mycoides*, (2) *Salmonella enterica*, (3) *Micrococcus luteus*, (4) *Escherichia coli*, (5) *Bacillus cereus*, (6) *Alcaligenes faecalis*, (7) *Proteus vulgaris*, (8) *Pseudomonas fluorescens*, (9) *Candida albicans*, (10) *Pseudomonas aeruginosa*, (11) *Staphylococcus aureus*, (12) *Pseudomonas libanensis*, (13) *Staphylococcus warneri*, (14) *Erwinia aphidicola*, (15) *Microbacterium foliorum*, (16) *Bacillus licheniformis*, (17) *Serratia plymuthica*, (18) *Rahnella aquatilis*, (19) *Erwinia aphidicola*, (20) *Bacillus endophyticus*, (21) *Leuconostoc mesenteroides*, (22) *Enterococcus casseliflavus*.

When estimating the antimicrobial activity of the strains studied, the following conclusion was reached: Bacteriocins of strain B884 exerted an inhibitory effect on two test cultures: *Staphylococcus warneri*, *Leuconostoc mesenteroides*; bacteriocins of strain B2430 – on 12 test cultures: *Escherichia coli*, *Alcaligenes faecalis*, *Candida albicans*, *Pseudomonas libanensis*, *Staphylococcus warneri*, *Erwinia aphidicola*, *Microbacterium foliorum*, *Bacillus licheniformis*, *Serratia plymuthica*, *Erwinia aphidicola*, *Bacillus endophyticus*, *Leuconostoc mesenteroides*; bacteriocins of strain B2455 – on 9 test cultures: *Escherichia coli*, *Alcaligenes faecalis*, *Pseudomonas libanensis*, *Erwinia aphidicola*, *Microbacterium foliorum*, *Bacillus licheniformis*, *Erwinia aphidicola*, *Leuconostoc mesenteroides*, *Enterococcus casseliflavus*.

DISCUSSION

Analysis of the results obtained in determining the intensity of bacteriocin production allowed to distinguish two strains: *Lactobacillus plantarum* B884 and *Lactobacillus paracasei* B2430, showed approximately the same results, but the quality of bacteriocin (antimicrobial activity relative to the strain *E. coli*) for strain *Lactobacillus paracasei* B2430 was higher.

For a more complete determination of the antimicrobial potential of bacteriocins synthesized by

strains of lactic acid bacteria and selection of the most effective strain, a minimum inhibitory concentration of strains with respect to the strain *E. coli*. The following results were obtained: the minimum inhibitory concentration for the strain *Lactobacillus plantarum* B884 was $1.0 \cdot 10^8$ CFU/ml, for strain *Lactobacillus paracasei* B2430 – $1.0 \cdot 10^6$ CFU/ml, for strain *Lactobacillus delbrueckii* B2455 – $1.0 \cdot 10^7$ CFU/ml. Thus, the lowest inhibitory concentration was observed for the strain *Lactobacillus paracasei* B2430.

When analysing the antagonistic activity of the bacteriocins of the studied cultures with respect to the 22 cultures of pathogenic strains, it was concluded that bacteriocins of the strain *Lactobacillus paracasei* B2430 possessed the greatest spectrum of antimicrobial activity, as they inhibited the development of most test cultures, compared with the remaining strains and, in addition, this strain demonstrated the best properties and the degree of inhibition of the growth of pathogenic strains.

Based on the results obtained in the study of the intensity of bacteriocin synthesis and their effectiveness, they concluded that bacteriocins of the strain *Lactobacillus paracasei* B2430 have the greatest antimicrobial activity as compared with other strains of lactic acid bacteria, which implies that this strain is promising for further study.

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Please cite this article in press as: Zimina M.I., Gazieva A.F., Pozo-Dengra J., Noskova S.Yu., and Prosekov A.Yu. Determination of the intensity of bacteriocin production by strains of lactic acid bacteria and their effectiveness. *Foods and Raw Materials*, 2017, vol. 5, no. 1, pp. 108–117. DOI: 10.21179/2308-4057-2017-1-108-117.

