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OPTIMIZATION OF ISOLATION AND PURIFICATION OF THE BACTERIOCIN FROM *ENTEROCOCCUS ITALICUS* ONU 547 BY ANION-EXCHANGE CHROMATOGRAPHY

Aim. *The aim of the work was to optimize isolation and purification of the bacteriocin from E. italicus ONU 547, which has biotechnological potential by low pressure anion exchange chromatography.* ***Methods.*** *Anion exchange chromatography by the BioLogic LP system with the cartridge Bio-Scale Mini Macro-Prep High Q was used for isolation of the bacteriocin from the culture liquid and for its partial purification. Two-step and three-step elution protocols were tested in order to increase yield of the bacteriocin and its purity.* ***Results.*** *The bacteriocin from culture liquid of E. italicus ONU 547 (320 AU/mL) was isolated and partially purified by anion-exchange chromatography with low-pressure system. The slightly alkaline hydrogen potential (pH = 8.0) of culture liquid and mobile phase was suitable for effective binding of the bacteriocin to the column with a positive charged matrix. The two-step (10% and 100% Buffer B with 1 M NaCl) elution procedure resulted in 4.4% yield of the bacteriocin, while three-step elution (10%, 50% and 100%) – 5%. The simplified procedure for bacteriocin isolation and purification is proposed.* ***Conclusions.*** *Low pressure anion-exchange chromatography with pH 8.0 of the mobile phase is effective for isolation of the bacteriocin directly from culture liquid of E. italicus ONU 547 and for its partial purification. Three-step elution procedure with 10%, 50% and 100% 1M NaCl leads to higher purity and yield of the bacteriocin.*

Key words: bacteriocin, Enterococcus italicus, anion exchange chromatography, peptide purification.

The problem of antibiotic resistance due to growing number of bacterial strains which are not sensitive to known antibiotics is one of the most important challenges in modern medicine worldwide which leads to 700000 deaths every year [1]. Among the infections caused by bacterial strains resistant to antibiotics, skin and wound infections, including with purulent complications are especially spread. The major pathogens are methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [8, 11].

The increasing problem of antibiotic resistance in pathogenic bacteria led to search their possible alternativei and ones of the most important among them are

bacteriocins from lactic acid bacteria (LAB) [1, 10]. Bacteriocins are antimicrobial peptides or proteins which are synthesized by ribosomes and show inhibitory activity against different species of bacteria [10].

In our previous research the bacteriocin from the strain *Enterococcus italicus* ONU 547 was partially purified and characterized. It showed promising inhibitory activity against some opportunistic pathogens, including wound infections. However, the tested classical four-step purification procedure, which included precipitation by 70% ammonium sulfate, ion-exchange chromatography and hydrophobic chromatography performed by Sep-Pak vacuum cartridges followed by reversed-phase high performance liquid chromatography, was not effective to obtain this antimicrobial compound [9]. Moreover, this procedure is complex and rather expensive. Unlike this approach, the ion-exchange chromatography alone looks much simpler and according to the literature data can result in high bacteriocin yield [10].

The information on bacteriocins from *E. italicus* species is scarce and only several publications can be found in scientific literature [5, 9]. To study bacteriocins from a theoretical and practical point of view their purification always is needed [10]. Moreover, one of the major drawbacks for usage of bacteriocins in clinical practice is low yield and high price of bacteriocin preparations. To make bacteriocin production possible in large-scale and cost-effective the known purification procedures have to be simplified [1].

Thus, the aim of this work was to optimize isolation and purification of the bacteriocin from *E. italicus* ONU 547, which has biotechnological potential by low pressure anion exchange chromatography

Materials and methods

The LAB strain *E. italicus* ONU 547 was used as a bacteriocin producer. This strain was isolated in our previous work from fermented plant material and for which the ability to produce a bacteriocin with a biotechnological potential was established [9].

As an indicator strain to determine the activity of the bacteriocin before and after purification, *Lactobacillus sakei* subsp. *sakei* JCM 1157 was used with known sensitivity to this antimicrobial compound [9].

The strains were stored at – 80 °C with 20% glycerol and activated by subculture in MRS (de Man, Rogosa and Sharpe) broth at 37 °C for 24 hours [3].

To obtain culture liquid from *E. italicus* ONU 547 with the bacteriocin the producer strain was inoculated in 200 mL and cultivated at 37 °C for 24 hours. After incubation, the culture was centrifuged at 10,000 × g (+4 °C, 10 min). The pH of the collected supernatant (CFS) was adjusted to 7.0 using 1 M NaOH [7].

The activity of the bacteriocin in the CFS was determined by the agar well diffusion assay against *L. sakei* subsp. *sakei* JCM 1157 (10^6 cells/mL) with two-fold serial dilutions in 0.2 M potassium phosphate buffer according to [6]. The same phosphate buffer and MRS broth were used as controls.

The amount of arbitrary units of the bacteriocin activity per mL (AU/mL) was determined by the following formula:

$$\frac{AU}{mL} = (1000 \div V) \times D,$$

where: V – volume of the sample poured into the well (50 μL), D – the last dilution that still results in the appearance of inhibitory activity against the indicator strain [6].

The isolation of the bacteriocin directly from the CFS and its purification were carried out using anion exchange chromatography with a BioLogic LP chromatography system and a Bio-Scale Mini Macro-Prep High Q Cartridge (BioRad, USA). Liquid chromatography was performed according to [4, 6] with significant modifications.

The Buffer A (20 mM Tris-HCl, pH 8.0) and the Buffer B (20 mM Tris-HCl, 1 M NaCl, pH 8.0) were used as the mobile phase at a flow rate of 1 mL/min [4]. The pH of the prepared buffers was adjusted to 8.0 with concentrated HCl. Then, the prepared solutions were filtered and degassed using an acetate filter and a Bunsen flask with a New Aspiret vacuum pump (Ca-Mi, Italy).

Thereafter, the pH of the CFS was adjusted to 8.0 using 1 M NaOH to provide the bacteriocin with a surface negative charge and filtered through a MillexGS MF-Millipore MCE Membrane syringe filter (Merck, Germany) with a pore diameter of 0.22 μm.

The chromatographic system was first washed with deionized water, then the Buffer A was passed through the system to measure its conductivity. After that, the column was activated with the Buffer B and equilibration was performed with the Buffer A. After stabilization the conductivity parameters, 2 mL of the filtered CFS were loaded into the sample loop and introduced to the column by the Buffer A. The same buffer was also used to wash the column

In order to optimize purification process the two elution protocols were tested. The first one was the two-step elution procedure with 10% Buffer B followed by 100%. The second elution protocol included three-step elution with 10%, 50%, and 100% Buffer B.

Each time after the increase in optical density, 1 mL samples were collected using a fraction collector into plastic microtubes and concentrated twice by evaporation using a TECHNE concentrator (Cole-Parmer, Germany) at 60 °C and nitrogen. As controls, CFS was used, which was loaded into the column, as well as Buffers A and B. After concentration, the samples were cooled, adjusted to neutral pH and tested for antimicrobial activity by the agar well diffusion assay, as described above. The active fractions after elution were pooled, two-fold serially diluted and tested for inhibitory activity to determine AU/mL, which depends on the protein concentration, as mentioned above. Yield of the bacteriocin was calculated based on sample volumes and AU/mL. The experiments were performed three times.

Results and discussion

Preparaition of CFS containing bacteriocin

At the beginning of the study, 200 mL of CFS from *E. italicus* ONU 547 with inhibitory activity 320 AU/mL against *L. sakei* subsp. *sakei* JCM 1157 were obtained. The CFS with inhibitory activity of the same level was obtained in our previous work [9] indicating stable bacteriocin synthesis of this enterococci strain, even after long storage.

The presence of inhibition against *L. sakei* subsp. *sakei* JCM 1157 in our experiments indicated only the activity of the antibacterial peptide, as was confirmed in our previous study by treatment of CFS with Proteinase K [9]. The AU/mL can allow suppose concentration of a bacteriocin without other sophisticated methods. This approach is standard in the study of LAB bacteriocins [6, 7]. It is known that bacteriocins, which are proteins or peptides by their chemical nature, exhibit antibacterial properties [2].

Isolation and purification of the bacteriocin by the ion-exchange chromatography with two-step elution

In order to optimize the purification of the bacteriocin from *E. italicus* ONU 547, the anion exchange chromatography method with the BioLogic LP chromatography system was used. The Bio-Scale Mini Macro-Prep High Q Cartridge column (BioRad, USA) was chosen as the stationary phase. Two elution options were tested: the first involved two steps, which were performed with 10% and 100% Buffer B, and the second involved three steps, performed with 10%, 50% and 100% of this solution.

As a result of the two-step elution procedure a chromatogram with four peaks was obtained (Fig. 1). The first peak, with a Rt of 0-5 min, corresponds to the stage of sample loading, the second and third with a time of 25 and 35 min – to the first elution (10% Buffer B), and the fourth, which was the largest peak and which appeared at 40 min of the experiment, corresponded to the second elution performed with 100% Buffer B.

The fractions were collected when increase in absorbance was observed on the chromatogram (green dots) and their antimicrobial activity was tested against *L. sakei* subsp. *sakei* JCM 1157 in order to detect the bacteriocin. It was found that

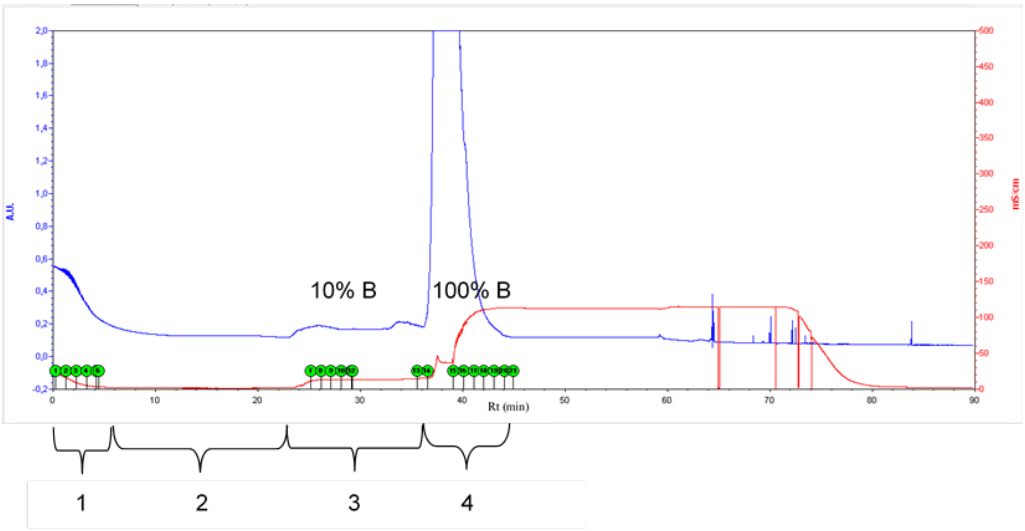

Fig. 1. Chromatogram of the purification process of the bacteriocin from *E. italicus* ONU 547 by anion exchange chromatography with two elution steps

Footnote: 1 – sample loading step, 2 – washing step, 3 – first elution step, 4 – second elution step

absorbance, conductivity

the highest antimicrobial activity and, accordingly, the highest concentration of the bacteriocin, were present in fractions №18 and 19 collected at 42 and 43 min of the experiment, respectively (Fig. 2). These fractions were collected at the second step of elution, which was carried out with 100% Buffer B. Therefore, only part of the fourth peak corresponds to the studied bacteriocin, and the rest of it is represented by other contaminant proteinaceous compounds, which were eliminated during the purification process.

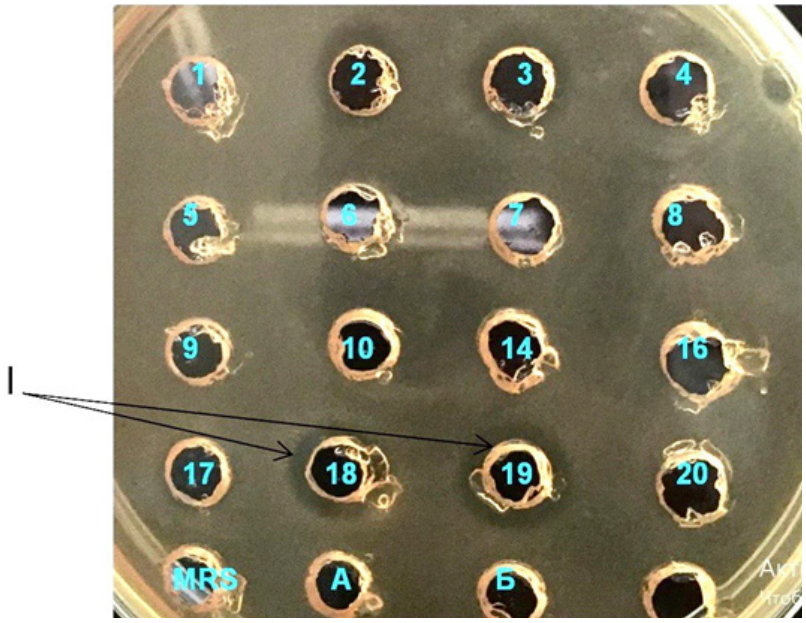

Fig. 2. Results of testing the antimicrobial activity of fractions obtained after anion exchange chromatography with two-step elution (10%, 100% Buffer B) against *L. sakei* subsp. *sakei* JCM 1157

Footnote: 1–6: sample loading step, 7–10: first elution step, 14, 16–20: second elution step; MRS – MRS broth, A – Buffer A, B – Buffer B (controls); I – zones of growth inhibition of the sensitive indicator strain

In addition, antimicrobial activity and, accordingly, the bacteriocin were absent in two peaks corresponding to the first elution step, which indicates that they are represented by other peptides or proteins. The absence of an increase in optical density during the washing step indicates a strong binding of this biomolecule under the study conditions to the column matrix by electrostatic interactions and, accordingly, the correct selection of the pH value of the CFS and buffers to provide the bacteriocin with a surface negative charge, which was slightly alkaline (8.0).

The two active fractions were pooled together resulted in a sample “EI” of 0.35 mL volume with 80 AU/mL.

Partial purification of the bacteriocin by the three-step elution procedure

The second variant of ion-exchange chromatography involved three elution steps by sequentially passing through the system 10%, 50%, and 100% Buffer B, As a result of the experiment, three peaks were observed on the obtained chromatogram, corresponding to the elution steps (Fig. 3).

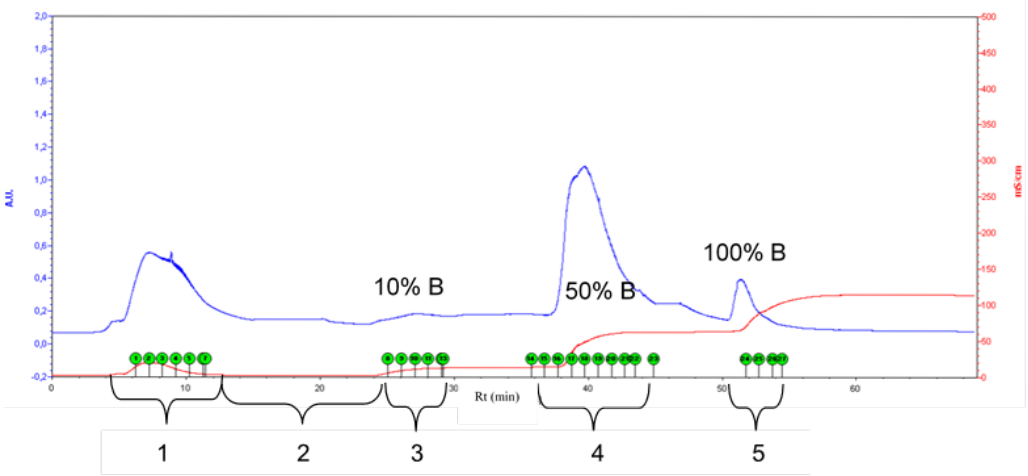

Fig. 3. Chromatogram of the purification process of the bacteriocin with three elution steps
Footnote: 1 – sample loading step, 2 – washing step, 3 – first elution step,
4 – second elution step, 5 – third elution step
absorbance, conductivity

The first elution peak, presented by compounds eluted from column by 10% Buffer B, appeared at 25–30 min of the experiment and was, as in the previous elution protocol, small. The second highest peak, appeared at approximately 40 min of the chromatography by passing 50% Buffer B, and the third peak, eluted from the column by 100% buffer B, had a retention time (Rt) of 50–52 min.

After testing the antimicrobial activity against *L. sakei* subsp. *sakei* JCM 1157, the bacteriocin was detected in fractions №18, 19 and 20, which corresponded to the main peak area of the second elution step (50% Buffer B). The Rt of the bacteriocin in this protocol was 40–42 min. The absence of antimicrobial activity in the first and third elution peaks indicate that they correspond to contaminants.

As in the previous elution procedure, the active fractions (№18, 19 and 20) were pooled in one tube. The resulted volume of the purified bacteriocin ("EII") after the three-step elution was 0.8 mL and the antimicrobial activity – 40 AU/mL, which were compared with the results of the two-step elution procedure (Table 1). The obtained results in this work are much better than in previous one, when the classic four-step purification procedure was applied, which resulted only in 20 AU/mL [9].

Table 1

Antimicrobial activity of the bacteriocin fractions obtained by anion exchange chromatography with two and three elution steps

Sample	Volume, mL	AU/mL	Yield, %
CFS	2	320	100
EI (two-step elution)	0.35	80	4.4
EII (tree-step elution)	0.8	40	5

It was found that the yield of purified antimicrobial compounds after the first variant of the experiment was 4.4%, and after the second – 5%. The results of this work are in agreement with the data of other scientists who also carried out the purification of enterococcal bacteriocins. Thus, it was reported that after a five-step purification procedure, which is much more complex, the yield of enterocins was 4% [6]. The further experiments on improvement of our results are now in progress.

To our knowledge, this is the first report on effective isolation and purification of a bacteriocin from the *E. italicus* species by anion-exchange chromatography. The purification procedure of other bacteriocins produced by the strain *E. italicus* GGN 10 was realized by precipitation with 60% ammonium sulfate followed by two steps of reversed-phase chromatography. However, yield of the purified compounds was not determined [5].

As for the purity of the obtained fractions, the bacteriocin sample obtained after anion exchange chromatography with three elution steps (EII) looked much clear compared to the control, which was used throughout the work as CFS. In addition, it should be noted that both obtained elution samples were transparent, unlike CFS, in which even after the filtration, undissolved particles were observed, which may be components of the MRS medium that were poorly dissolved as well as aggregated peptides. In our further work we plan to perform protein electrophoresis to additionally assess the purity of the bacteriocin after mentioned chromatography procedures.

Thus, the purification process of the bacteriocin from *E. italicus* ONU 547 was optimized using anion exchange chromatography with the BioLogic LP system and the Bio-Scale Mini Macro-Prep High Q Cartridge column from BioRad (USA). As a result of the tested two protocols of step elution it was found that the best bacteriocin yield, which was 5%, and the highest level of purity were observed after using a three-step elution, which involves the step passage through the system of 10%, 50% and 100% Buffer B containing 1M NaCl. This isolation and purification procedure is simple, does not require preliminary concentration and purification stages, and can be used to obtain purified samples of the bacteriocin from *E. italicus* ONU547 for its further study and application in biotechnology.

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ОПТИМІЗАЦІЯ ВИДІЛЕННЯ ТА ОЧИСТКИ БАКТЕРІОЦИНУ *ENTEROCOCCUS ITALICUS* ОНУ 547 ЗА ДОПОМОГОЮ АНІОНООБМІННОЇ ХРОМАТОГРАФІЇ

Резюме

Мета. *Метою роботи була оптимізація виділення та очищення бактеріоцину з E. italicus ОНУ 547, який має біотехнологічний потенціал, за допомо-*

гою аніонообмінної хроматографії низького тиску. ***Методи.*** *Для виділення бактеріоцину з культуральної рідини та його часткового очищення використовували аніонообмінну хроматографію, що здійснювали за допомогою системи BioLogic LP з картриджем Bio-Scale Mini Macro-Prep High Q. Для збільшення виходу бактеріоцину та його чистоти було випробувано протоколи двох крокової та трьох крокової елюції.* ***Результати.*** *Бактеріоцин з культуральної рідини E. italicus ОНУ 547 (320 ВО/мл) було виділено та частково очищено за допомогою аніонообмінної хроматографії з системою низького тиску. Слаболужний водневий потенціал (pH=8,0) культуральної рідини та рухомої фази був придатним для ефективного зв'язування бактеріоцину з позитивно зарядженим матриксом колонки. Двоступенева процедура елюції (10% та 100% буферу В з 1 М NaCl) призвела до виходу бактеріоцину 4,4%, тоді як триступенева елюція (10%, 50% та 100%) – 5%. Запропоновано спрощену процедуру виділення та очищення бактеріоцинів.* ***Висновки.*** *Аніонообмінна хроматографія низького тиску з pH 8,0 рухомої фази є ефективною для виділення бактеріоцину безпосередньо з культуральної рідини E. italicus ОНУ 547 та для його часткового очищення. Триступенева процедура елюції з 10%, 50% та 100% 1 М NaCl призводить до вищої чистоти та виходу бактеріоцину.*

Ключові слова: бактеріоцин, Enterococcus italicus, аніонообмінна хроматографія, очищення пептидів.

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