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## ОПТИМИЗАЦИЯ СОСТАВА ПИТАТЕЛЬНОЙ СРЕДЫ МИКРООРГАНИЗМОВ КАК ПОДХОД К ВЫДЕЛЕНИЮ БАКТЕРИОЦИНОВ

Антимикробные пептиды бактериального происхождения можно рассматривать как эффективные пищевые консерванты, а также терапевтические средства. Первые трудности в изучении этих веществ начинаются на стадии изоляции бактериоцина от культурной среды производителя микроорганизма. Представлен простой и доступный подход к оптимизации изоляции бактериоцинов от метаболитов микроорганизмов.

В этом исследовании штамм *E. faecium* ICIS 7 был использован в качестве производителя бактериоцина. Бактерии культивировались на четырех различных средах, состоящих из двух коммерческих сред и двух полусинтетических. Выделение бактериоцинов проводили обращенно-фазовой жидкостной высокоэффективной хроматографии. На первом этапе были оценены параметры роста бактерий в различных средах и полуколичественная оценка производства бактериоцина. На втором этапе получены и сопоставлены хроматографические профили. Наконец, было показано, что использование простой среды, состоящей только из экстракта дрожжей, позволяет производить бактериоцины и впоследствии упростить их хроматографическую очистку.

Таким образом, можно сделать вывод, что экстракт дрожжей является ингредиентом, доступным большинству лабораторий, в то время как компоненты для приготовления химически определенной среды по-прежнему являются достаточно дорогостоящими химическими реагентами (аминокислоты, нуклеиновые кислоты и др.). Применение дрожжевого экстракта для выращивания и производства бактериоцинов значительно снижает затраты на изоляцию целевого полипептида.

**Ключевые слова:** бактериоцины, минимальная среда, энтерококки

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## OPTIMIZATION OF A CULTURE MEDIUM COMPOSITION AS APPROACH TO ISOLATION OF BACTERIOCINS

Antimicrobial peptides of bacterial origin can be considered as effective food preservatives, as well as therapeutic agents. The first difficulties in the study of these substances begin at the stage of bacteriocin's isolation from the culture medium of the producer microorganism. A simple and affordable approach for optimizing isolation of bacteriocins from metabolites of microorganisms is presented.

In this study a strain of *E. faecium* ICIS 7 was used as bacteriocin producer. Bacteria were cultured on four different media comprising two commercial medium and two semisynthetic. Isolation of bacteriocins was carried out by reversed-phase liquid high-performance chromatography. At the first stage, the bacterial growth parameters on various media were estimated and bacteriocin's production was evaluated semi-quantitatively. At the second stage chromatographic profiles were obtained and compared. Finally it was shown that using a simple media consisting of the yeast extract only, makes it possible to produce bacteriocins and subsequently simplify their chromatographic purification.

Thus, it can be concluded that the yeast extract is an ingredient available to most laboratories, while the components for the preparation of the chemical-defined medium are still quite expensive chemical reagents (amino acids, nucleic acids etc.). The use of a yeast extract for the cultivation and production of bacteriocins significantly reduces the cost of isolating the target polypeptide.

**Key words:** bacteriocin, simple medium, chemically defined medium.

Resistance to antibiotics as a biological phenomenon significantly complicates the treatment of infections caused by pathogenic micro-organisms. By now, there is a trend when available drugs are no longer effective [1]. The leading reasons for this situation are the uncontrolled prescription of drugs,

as well as the absence of new significant developments in this direction. In this regard, the search for new effective antimicrobial substances seems to be a timely and in demand research direction. One of the directions is the isolation and study of the biological effects of antimicrobial peptides (AMPs), which

are synthesized by a wide range of living organisms. Antimicrobial peptides are a short sequence of amino acids that have amphiphilic properties and are predominantly positively charged [2]. These features allow them to effectively interact with the surface structures of bacterial cells with subsequent disruption of their normal physiology [3].

Currently, more than 2,000 different antimicrobial peptides are known [4]. The circle of organisms being capable of producing AMP includes a variety of taxa, which indicates the important role of AMP in the system of the innate immunity of the organism, without which survival in a medium full of various micro-organisms is impossible [5]

More than 2730 different antimicrobial peptides have been isolated and characterized, of which 10% are bacteriocins, 12% are plant and more than 75% are of animal origin [4]. However, unlike antimicrobial peptides produced by eukaryotic organisms, bacteriocins are the least studied group of such substances.

Unlike peptides of animal origin, bacteriocins are a much more difficult object to study, because bacteria synthesize active peptides in ultra-small amounts, while extraction and purification from microbial metabolites is also a difficult task. The main problems of isolating a target substance are related to the fact that the bacteria grow and produce bacteriocins in multi-component culture media saturated with various proteins and peptides. To obtain a homogeneous form of bacteriocins, a variety of isolation and purification methods, such as precipitation, cation-exchange chromatography and multiple reversed-phase HPLC are used.

These purification methods require expensive equipment at each step, and may result in a significant loss of desired product [6]. An increase in the effectiveness of liquid chromatography methods, as well as a reduction in the number of purification steps may be due to the cultivation of producers on media with a minimum content of proteins and other hydrophobic components that impede the release of bacteriocins [7]. Most bacteriocin-producers are lactobacilli, which are quite demanding in the composition of the nutrient medium. Comparison of different media showed that de Man Rogosa Sharpe (MRS) medium is the most optimal for bacteriocin production [8], but the chromatographic purification of bacteriocins from such a complex medium will be hindered by an abundance of proteins, especially casein hydrolyzate. The

number of purification steps will include from three [9] to six [10]. One of the approaches to optimizing the procedure for the isolation of bacteriocins is the use of simple media containing a minimal list of ingredients [11]. One of the first experiences of using simple media was obtained in 1957 when Arnoldi purified megacin from *Bacillus megaterium* growing in a defined medium in the presence of an adequate concentration of manganese [12].

To date, there has been some experience of using a chemical-defined medium (CDM) for the cultivation of LAB-bacteria and purification of bacteriocins [13]–[16]. CDM-grown bacteria retain the ability to produce antimicrobial peptides, while only one step is required to purify bacteriocin. On the other hand, the cost and availability of components for CDM such as a chemically pure amino acids and purine/pyrimidine bases makes practical use are difficult.

In this regard, the aim of this work was to investigate the possibility of using a simple medium for producing different bacteriocins and comparisons with a complex commercial medium. Thus, the main goal of this work was not related to increasing bacteriocins production, but deals with optimization of the chromatographic purification of them.

**Materials and Methods.** *Bacterial strains, growth conditions and chromatographic procedures*

In the investigations of growth and bacteriocin production, two bacteriocin-producing strains – *E. faecium* ICIS 7 and *K. pneumoniae* ICIS 1160 – were used. Bacteria were grown on four media of differing components (Table 1). Bacteria were incubated in 50 ml flacons at 37 °C for 24–36 hours. The dynamic of bacterial growth was assessed by reading and plotting the absorbance data at 620 nm obtained by spectrophotometer IEMS MF (Lab-systems, Finland). Evaluation of the bacteriocin content was performed by isolating an aliquot of the culture medium followed by centrifugation (20 min, 4 °C, 9000 g) and then filtering through a Durapore® PVDF filter with pore size 0.22 µm (Millipore). Activity of bacteriocins was expressed in arbitrary units per millilitre, calculated according to formula [17] (1):

$$AU = (1000 / V) * D$$

where, V – supernatant volume,

D – dilution factor.

At the next step the desalting of the culture medium was performed using a reversed-phase low-pressure chromatography on a Brownlee Aquapore RP-300 column (PerkinElmer, USA) equilibrated with solvent A (10% acetonitrile (Merck, Germany) in ultra-purified water (18 ohms, Milli-Q, Millipore) with 0.1% trifluoroacetic acid (TFA). Elution was performed using the solvent B (80% acetonitrile in ultra-purified water with 0.1% TFA) followed by lyophilization to withdraw a residual quantity of TFA and concentration. The obtained desalted extract was tested to reveal the antibacterial properties. The lyophilized mass was resuspended in ultrapure water and applied to analytical column C<sub>18</sub> Luna (250 x 4.5 mm, Phenomenex, USA) integrated into a high-performance liquid chromatography (HPLC) system (Knauer SmartLine 200, Knauer, Germany). Elution was performed using the solvent B (80% acetonitrile in ultrapure water with 0.1% TFA) in a linear gradient according to the scheme: 0–70% in 60 min, at a flow rate of 0.7 ml/min. Absorbance was detected at 220 nm.

*Determination of activity by agar well diffusion assay*

Determination of bactericidal properties of the culture medium and HPLC-fractions were performed using an agar well diffusion assay, as described earlier [18].

Briefly, the micro-organism indicators *L. monocytogenes* 88 BK or *E.coli* MG1655 were cultured for 18 h in Schaedler broth (HiMedia, India), after which 50 µl of the bacterial suspension

(containing ~ 10<sup>7</sup> CFU ) was mixed with 10 ml of soft (0.5%) Schaedler agar and placed immediately over the Petri dish, which was previously overlaid with 1.5% Schaedler agar plate. Solidified agar plates were punched with a 5 mm diameter flame-sterilized cork borer and the twofold serial diluted samples were transferred into the wells. After incubation at 37 °C overnight, inhibitory areas were observed.

**Results and Discussion.** For the experiment, two commercial media with different content of proteins and peptides were chosen. The Schaedler medium is characterized by a complex of different components, which are used to isolate bacteria from the intestinal microbiota.

As sources of nutrition, the Schaedler medium (HiMedia, India) includes hydrolysates of casein, soy, meat, yeast extract and glucose (Table 1). The LB broth (Miller, Merck, Germany) is a widely used broth for cultivation of bacteria in the lab. This broth is nutrient-rich and contains peptides, amino acids, water-soluble vitamins, and carbohydrates. For the preparation of the minimal medium (MM1) we used a yeast extract only manufactured by Becton Dickenson (BBL); in another variant a glucose was added (MM2).

The first tested strain was *E. faecium* ICIS 7, which belongs to the LAB group of bacteria, which are characterized by a complex food preference. In this connection, to isolate enterococci from the human and animal intestines a multicomponent medium is used such as the Schaedler medium. The

Table 1 – Composition of the commercial complex medium and the laboratory simple medium

| Components                      | Content of components, g L <sup>-1</sup> |          |      |      |
|---------------------------------|--|----------|------|------|
|                                 | Schaedler-broth                          | LB-broth | MM 1 | MM 2 |
| Casein enzymic hydrolysate      | 5.67                                     | 10       | -    | -    |
| Proteose peptone                | 5.0                                      | -        | -    | -    |
| Papaic digest of soyabean meal  | 1.0                                      | -        | -    | -    |
| Yeast extract                   | 5.0                                      | 5.0      | 10   | 10   |
| Dextrose                        | 5.83                                     | -        | -    | -    |
| Sodium chloride                 | 1.67                                     | 5.0      | -    | -    |
| Dipotassium hydrogen phosphate  | 0.83                                     | -        | -    | -    |
| Tris hydroxymethyl aminomethane | 3.0                                      | -        | -    | -    |
| L-Cystine                       | 0.4                                      | -        | -    | -    |
| Hemin                           | 0.01                                     | -        | -    | -    |
| Glucose                         | 5.83                                     | -        | 5    | -    |

growth of this strain on Schaedler broth was characterized by a prolonged lag-phase during which bacteria adapted to the polycomponent medium (Fig. 1 a). From the middle part of the log-phase, the synthesis of bacteriocin begins (Fig. 1 b). The maximum amount of bacteriocin have accumulated in the stationary phase at the end of cultivation. It is known that production of bacteriocins is a quorum-dependent phenomenon [19], well documented for other strains [20]. So, maximum A.U. values ( $6.4 \times 10^3 \text{AU ml}^{-1}$ ) were revealed in the stationary phase when the number of cells is highest.

In turn, cultivation of *E. faecium* ICIS 7 on LB medium are characterized by certain features. In particular, the bacteria adapted quickly to the medium, which was expressed by a lag-phase reduced by half if compared to that on Schaedler medium. The exponential phase was finished at 6 hours of cultivation. At the same time, the optical density of the suspension in the stationary phase was twofold less in comparison with the population of cells in the Schaedler medium.

Production of bacteriocin began when the bacterial population reached the maximum density. At the same time, bacteriocin was synthesized in smaller amounts ( $1.6 \times 10^3 \text{AU ml}^{-1}$ ) if compared with cultivation on the Schaedler medium (Fig.1 b).

In turn, the use of a yeast extract solution as a nutrient medium revealed a number of interesting features. Firstly, the growth parameters of *E. faecium* on this medium with glucose were comparable with the growth on a commercial LB medium, while

growth in the same medium without glucose was less productive.

Secondly, suppression of bacteriocin production on the medium containing yeast extract and glucose (MM 1) was surprising (Fig. 1 b), while biomass production was comparable with that LB broth (Fig 1. a). Earlier, a similar inhibition effect of glucose on bacteriocin production was shown by the cultivation of various LAB bacteria [20].

Activity of bacteriocin produced on MM 2 was detected but to a lesser degree ( $0.4 \times 10^3 \text{AU ml}^{-1}$ ) if comparing with a commercial broth (Fig.1 b).

After the accumulation of bacteriocin in the medium, it must be isolated. Often, pre-sample preparation followed by multi-step chromatography are performed. Ultimately, these manipulations increase the costs and reduce the amount of the target product. In this work, desalted extracts of various media obtained by culturing bacteria were applied on a chromatographic column filled with the  $C_{18}$  reversed-phase. In each case, identical elution parameters were used. As a result, four chromatographic profiles were obtained (Fig. 2).

Thus, when the metabolites of bacteria grown on the Schaedler broth were divided, the obtained chromatographic profile was characterized by 40 broadened peaks (Fig. 2a); the profile of the LB medium containing bacterial metabolites was formed by 32 fractions (Fig. 2b).

In turn, the chromatographic profile of the MM1 and MM2 medium consisted of only 19 and 25 fractions, respectively (Fig. 2 c, d). The raw

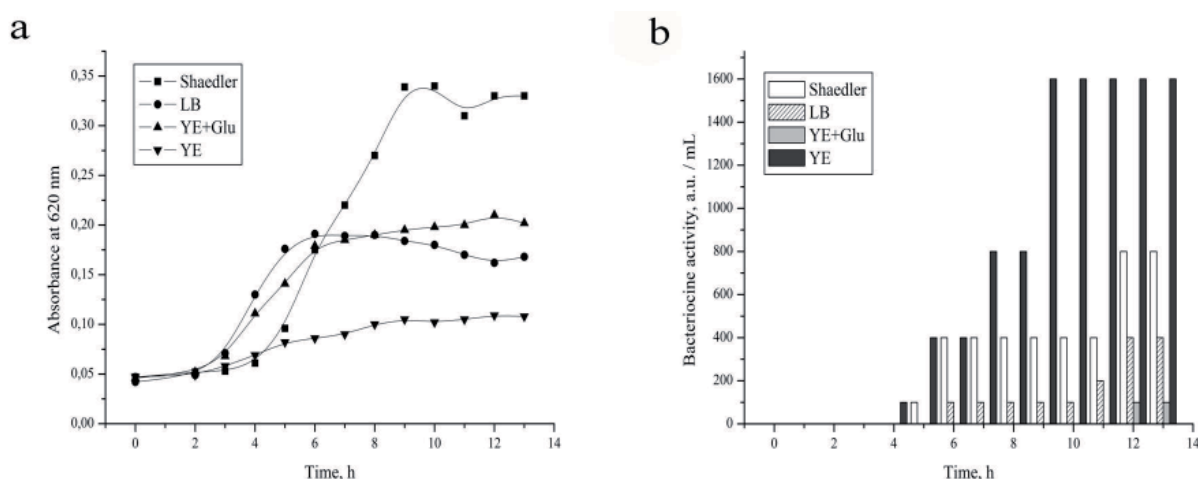


Figure 1 – Changes in growth (a) and bacteriocin production (b) by *E. faecium* ICIS 7 cultivated on different media. Growth (a) was followed by changing the optical density ( $OD_{620}$ ), production of bacteriocin (b) was determined by the spot-on-lawn assay and expressed in arbitrary units ( $\text{AU ml}^{-1}$ ).

yeast extract contained a variety of proportions of nucleic acids, polysaccharides and peptides. According to the manufacturer the used yeast extract is composed of peptides with a molecular mass < 250 Da (70 %) and 0.5–2.0 kDa (20 %). Thus, using the yeast extract only as a nutrient base allowed a substantial increase the efficiency of separation of *E. faecium* metabolites by reducing the load of proteins and peptides at the stage of culturing.

Thus, we have shown that yeast extract as a nutrient base is sufficient to cultivate various bac-

teria and obtain an accumulation of bacteriocins in the culture medium. The undoubted advantage of this approach is the isolation of bacteriocin by one-step reversed-phase liquid chromatography. Such an approach consisting of the optimization of a culture medium composition to cultivate and purify bacteriocins was previously tested on *E. faecium* B9510. This bacteria was cultivated on a medium containing crystalline amino acids as a nitrogen source, which enabled the use of ultrafiltration to isolate Enterolysin A [21].

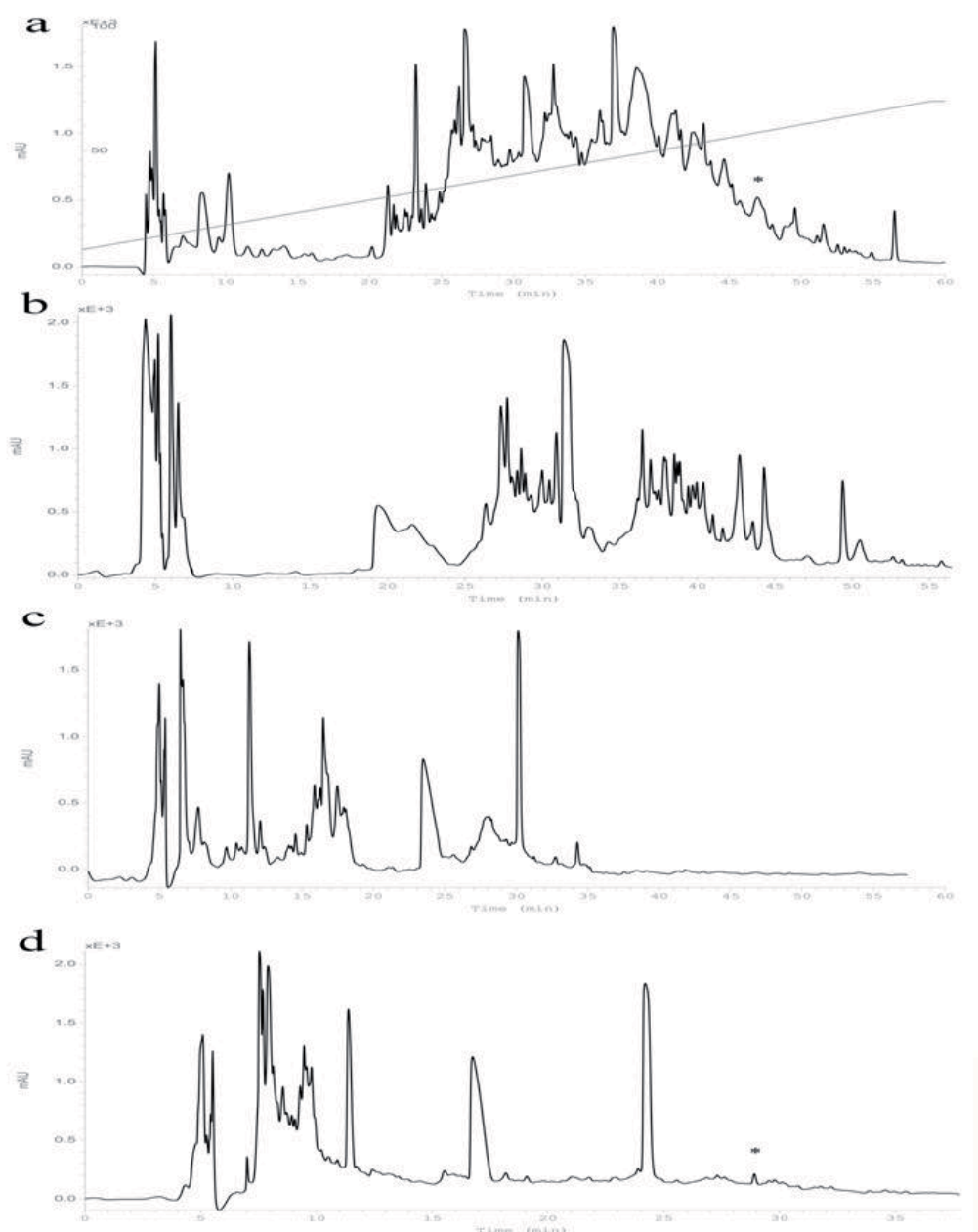


Figure 2 – The profiles of analytical RP-HPLC of various culture media after cultivation of *E. faecium* ICIS 7. a – Schaedler broth; b – LB broth; c – MM1; d – MM 2. Active fraction are marked by asterisk and grey color.



**Conclusion.** Thus, it can be concluded that the yeast extract is an ingredient available to most laboratories, while the components for the preparation of the chemical-defined medium are still quite expensive chemical reagents (amino acids, nucleic acids etc.). The use of a yeast extract for the cultivation and production of bacteriocins significantly reduces the cost of isolating the target polypeptide.

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