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ABSTRACT

of the dissertation submitted for the scientific degree of
Doctor of Philosophy

BIOTECHNOLOGICAL BASES OF THE SYNTHESIS OF PROTEIN ORIGIN ANTIBIOTICS AT THE BACTERIA OF THE GENUS BACILLUS

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INTRODUCTION

Relevance and degree of the completion of the topic. Certainly, various antibacterial substances play an important role in human life. Antibacterial, antifungal and antiviral antibiotics every day helps to save lifes of the thousands of sick people and other creatures - plants and animals. The number of antibiotics that are prescribed against bacteria and viruses is increasing every year, new medications become more effective and has wider spectrum of action. While the first antibiotics were obtained mainly from microscopic fungi, then more affordable and more efficient chemical synthesis began. However, "*the uncontrolled, sometimes unjustified and widespread use of antibiotics has led to the emergence of bacterial resistant to their effects. Novadays antibiotic-resistant strains are the main cause of hospital infections*"[1,2,3]. Antibiotics, as chemicals foreign to the human body, has the features to cause complications in the blood and internal organs. Classical antibacterial agents also led to other complications. They cause allergic reactions in some people and inhibits the immune system. Because chemical antibiotics have a wide spectrum of action, they destroy not only pathogenic microbes, but also the normal microflora of the intestine and mucous membranes which lead to dysbacteriosis and mycoses.

Thus, the search for new types of antibacterial drugs has emerged as an important issue. In this regard, antibacterial substances of natural biological origin, that is, bacteriocins, attract great attention of scientists.

In this regard, antibacterial substances of natural biological origin, that is, bacteriocins, have become a special focus of scientists.

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[1] Djordjevic, Z.M. Previous antibiotic exposure and antimicrobial resistance atterns of Acinetobacter spp. and Pseudomonas aeruginosa isolated from patients with nosocomial infections / Z. M. Djordjevic, M. M. Folic, S.M. Jankovic // Balkan Medical Journal, - Kragujevac: - 2017. 34(6), - p.527- 533.

[2] Boev, C., Kiss, E. Hospital-acquired ınfections: current trends and prevention // - Pittsburg: Critical Care Nursing Clinics of North America, Pittsburg. – 2017. 29(1), - p. 51- 65.

[3] Овчинников, Р.С. Этиопатогенез современных инфекций // - Москва: Vetfarma, - 2015. ч. 2, №3, - с. 40- 42.

Unlike antibiotics, these protein substances are not characterized by negative properties typical to conventional antibiotics. The small number of microorganisms involved to research in this field, the limitation of biological productivity of the producents for certain reasons, as well as the fact that several challenges related to the synthesis of bacteriocins have not been clarified until the end, make it necessary to conduct research in this destination. Study of characteristics of bacteriocins, selection of bacteriological media for cultivating producent bacteria, determination of optimal conditions and adaptation of synthesis methods to industrial conditions constitute the relevance of the dissertation research.

The aims and tasks. The aim of the presented research is to synthesize antibacterial substances from bacteria of the genus *Bacillus* (including *B. subtilis* ATCC 6633, *B. cereus* ATCC 14579 and natural *Bacillus* strains) by efficient, affordable, and production-applicable methods.

With purpose to achieve the goal, the fulfillment of the following tasks was considered appropriate:

1. Study of the producent characters and antibacterial properties of the selected *Bacillus* strains (including *B. subtilis* ATCC 6633, *B. cereus* ATCC 14579 and natural *Bacillus* strains)

2. Synthesis of the antibacterial substances of protein origin from bacteria of the genus *Bacillus* (*Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 6633 collection strains and natural *Bacillus* strain isolated from Kura River) using selected methods.

3. Measurement of chemical purity, concentration, and molecular mass of the bacteriocins synthesized from selected *Bacillus* bacteria and determination of their antibacterial properties.

4. İnvestigation of the genetic base of subtilin, subtilisin and polymyxin bacteriocins in the genetic material of *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 6633 collection strains and the natural *Bacillus* strain isolated from Kura River.

Research methods. The accuracy of the results obtained was confirmed by the application of biotechnological, bacteriological (manual and automatic) and genetic methods that meet modern standards, and the repeatability of all experiments performed. The

purity of the bacterial strains and reagents used for the experiments, the accuracy supported by the calibration of the devices, and the quality control of the nutrient media and tests also determine the honesty of the results obtained.

The main provisions of the dissertation submitted for defense.

1. Bacteria of the genus *Bacillus* (*Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 6633 collection strains and natural *Bacillus* strain isolated from Kura River) has bacteriocin producent properties, that is why there is opportunity to use them as effective producents for synthesis of the protein origine antibacterial substances.

2. The fact that the production of bacteriocins from *Bacillus* bacteria is complex, requires significant investment and special conditions, which necessitates the identification of simpler and more cost-effective synthesis conditions.

3. The bacteriostatic effect of *Bacillus* bacteria is associated with the bacteriocins they secrete.

4. The bacteriocins secreted by the *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 6633 collection strains and natural Bacillus strains isolated from the Kura River are associated with the presence of the corresponding genes in their genome.

Scientific novelty of the research. During the four years of research conducted in the laboratory, scientific and laboratory methods were modified, and their optimal variants were determined. In particular, convenient and simple methods were selected for obtaining antibacterial substances from *Bacillus* bacteria (both collection strains and strains isolated from the water of the Kura River): suspensions of 72-hour bacterial cultures were shaken for 18 hours on the blood roller, then centrifuged. Protein-containing bacteriocins were precipitated from the collected supernatant with acetone.

An original and optimal method was selected to purify *Bacillus* bacterial suspensions from alive bacteria. For this, biotechnological methods of centrifugation, high-temperature pasterization and bacterial filtration were compared, and the centrifugation method was

preferred. It was found that bacteriocins are secreted more actively by *Bacillus* bacteria precisely after long-term cultivation. This result has general meaning and can be used to obtain various protein-based substances from other bacteria.

For the first time, the process of "aging" in *Bacillus* bacteria was observed and described under a light microscope. Distinctive features were found in the morphological characteristics, arrangement, and spore formation of one-and three-day-old bacterial cells of the genus *Bacillus*.

Bacteriocins were extracted from *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 14579, and *Bacillus* strains isolated from the Kura River, their concentration and molecular mass were measured, and the spectrum of antibacterial properties was determined. The antibacterial properties of the synthesized new bacteriocins were tested and confirmed. The antibacterial properties of the new bacteriocins and their corresponding bacterial cultures were compared. Their inhibition against the same bacteria was confirmed.

Genetic tests were conducted for *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 14579, and *Bacillus* strains isolated from the Kura River. The genetic basis of the subtilin bacteriocin, namely the spaS gene, was found in the DNA of the *Bacillus subtilis* ATCC 6633 strain, the subtilisin gene sboA was found in the DNA of the *Bacillus cereus* ATCC 14579 strain, and the pmxE gene was found in the DNA of the *Bacillus polymyxa* strain, extracted from Kura River.

Theoretical and practical significance of the research. The studies conducted within the framework of the scientific work are important both theoretically and practically. The methods presented in the dissertation are suitable for determining the antagonism not only of bacteria of the genus *Bacillus*, but also of other bacteria. These methods can quickly stimulate the discovery and synthesis of numerous producers. On the other hand, they can create an opportunity for the synthesis of bacteriocins in conditions requiring low investment. The synthesis of new bacteriocins carried out in laboratory conditions can be easily applied to production. The proposed method can help provide the population with safe and selective natural antibiotics. Bacteriocins will help in the treatment of

both humans and animals, as well as plants. The optimization of bacteriocin synthesis methods and their adaptation to industrial processes will give impetus to new research.

Publication, approval and application of the dissertation. 17 works on the topic of the dissertation have been published and their results have been reported at the International Symposium "The 5th International Symposium and School for Young Scientists on Physics" (Russian Federation, Moscow, 2020), the International Scientific and Practical Conference of the Azerbaijan Veterinary Scientific Research Institute (Azerbaijan Republic, Baku, 2019), the "3rd Symposium Dedicated to World Health Day" held at Khazar University (Azerbaijan Republic, Baku, 2019), twice at the LXIII International Scientific Conference on "Development of Science in the 21st Century" (Ukraine Republic, Kharkov, 2020), the AGRO International Agricultural Conference (Azerbaijan Republic, Baku, 2022) and the "VI International European Conference" (Romania Republic, Bucharest, 2022).

The organization where the dissertation was carried out. The dissertation work was carried out in the microbiological biotechnology laboratory of the Institute of Microbiology of the Ministry of Science and Education of the Republic of Azerbaijan. Few experiments were conducted in the Veterinary Laboratory of the State Veterinary Supervision Service of the Republic of Azerbaijan.

The structure and volume of the dissertation. The dissertation consists of an introduction, 6 chapters (literature summary - I, materials and methods - II, experimental part - III - VI), final analysis of research results, conclusions, lists of literature and abbreviations used in the dissertation and applications, which is a total of 245,052 signs.

CHAPTER I
BACTERIA OF THE GENUS BACILLUS AND THEIR BACTERIOCINS

In section 1.1 of the dissertation, the characteristics of *Bacillus* bacteria such as "*rapid and easy reproduction, productivity, safety,*

resistance to adverse environmental factors"[4] were shown for the purpose of determining potential bacteriocin characteristics. In section 1.2, the "*tinctorial and cultural properties of the strains, their colony morphology, distribution in the environment, safety and other confirmatory identification characteristics of Bacillus cereus ATCC 14579 and Bacillus subtilis ATCC 6633 selected for bacteriocin synthesis*"[5,6] were analyzed. In section 1.3, information on the chemical structure, synthesis, genetic basis of existing bacteriocins of *Bacillus* bacteria and the electronic databases covering them ("BACTIBASE"[7], "NucleBact"[8] and BAGEL) were analyzed for the purpose of planning the scientific work.

CHAPTER II

MATERIALS AND METHODS OF THE RESEARCH

2.1. Bacterial strains used for the synthesis of bacteriocins

There are three bacteria - *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC6633 collection strains and *Bacillus polymyxa* obtained from the nature of Azerbaijan, in particular, from the water of the Kura River were selected for syntheses of bacteriosins - antibacterial substances of protein origin. During the preparation of the bacterial strains for the experiments, it was confirmed that they

[4] Еременко, Е.И. «Bacillus cereus» - проблемы идентификации и таксномии // - Ставрополь: Медицинский вестник Северного Кавказа, - 2008. № 3, с. 57-59.

[5] Чубенко, Г.И. Методы идентификации бактерий / Г.И.Чубенко. - Благовещенск: Амурская Государственная Медицинская Академия Министерства Здравоохранения России , - 2018. - 44 с.

[6] El-mishad, M.A. Manual of practical microbiology / M.A. El-mishad. - Cairo: Cairo University, - 2015. - 149 p.16.

[7] Hammami, R. [et al.] BACTIBASE second release: a database and tool platform for bacteriocin characterization: [Electronic resource]/BMC Microbiology.-California,-2010.

[8] Jong, A., Hijum, A.F., Jetta, J.E. BAGEL: a web-based bacteriocin genom meaning tool: [Electronic resource] / Nucleic Acids Research. – Oxford, 2006.

were viable and suitable for research, that is, pure, uncontaminated cultures. Their morphological, cultural and tinctorial "*identification was carried out according to the recommendations of the ABIS database system of India (Kerala)*"[9].

2.2. Methods applied for the synthesis of bacteriocins

The accuracy of the research results was confirmed by the numerous experiments conducted using bacteriological, biotechnological (physical, chemical) and genetic methods the numerous experiments conducted using bacteriological, biotechnological (physical, chemical) and genetic methods that meet modern standards.The antagonism of bacteria of the genus *Bacillus* and their bacteriocins were tested "*by various known, but modifiyed bacteriological methods*"[10,11] and at the same time, effective methods were used in the experiments. Biotechnological methods such as dissolving bacterial cultures in sterile physiological solution, shaking them on a blood roller, used. Since the aim of the study was to develop a methodology suitable for the industrial production of bacteriocins, the simplest and most convenient and, spinning in a centrifuge and separating the active substance from the supernatant with acetone were tested to find their most optimal options. Within the framework of the study, biotechnological physical methods such as centrifugation and obtaining the supernatant, passing through bacteriological filters and heating to purify suspensions from live bacteria, were compared, and, as a result, centrifugation was preferred. In order to determine the amount and concentration of proteins in sterilized solutions, a "total protein" test was performed on

[9] Paenibacillus (Bacillus) polymyxa: [Electronic resource] / Advanced Online Identification Software Encyclopedia, Activity-Based Information System (ABIS). - Kerala, 2022.

[10] Иркитова А.Н., Яценко, Е. С. Оптимизация метода определения антагонистической активности пробиотических бактерий // Алтай: Технологии пищевой и перерабатывающей промышленности АПК - продукты здорового питания, - 2017. № 5, - с. 114-116.

[11] Tagg, J.R. Assay system for bacteriocins /J.R.Tagg. - Cambridge: Applied Microbiology, - 1971. 21(5), - p. 943.

a COBAS e400 automatic biochemical analyzer, and the protein concentration was measured spectrophotometrically. The new protein-based antibacterial agents obtained solution were precipitated with cold acetone. To determine which proteins were precipitated, the molecular mass of all three protein-derived substances was measured and calculated polyacrylamide using gel (PAAG) electrophoresis.When proteins and calibrators (standards) run in one direction from "negative" to "positive" in an electromagnetic field, the distance they passed is directly dependent on the molecular size (mass) and electric charge. Using a correlation graph between mass and distance, the molecular mass of the synthesized proteins was measured and compared with the molecular mass of other proteins. As a result, it was found that the molecular mass of *Bacillus cereus* ATCC 14579 *Bacillus subtilis* and natural *B. polymyxa* proteins is equal to the molecular mass of subtilin, subtilisin, and polymyxin proteins, respectively. Purit of the synthesized proteins was determined using ng spectrophotometry. In order to once again confirm that these proteins are indeed subtilin, subtilisin and polymyxin, the genes responsible for the synthesis of antibacterial substances were investigated in the genetic material of the corresponding bacterial strains, i.e., in the extracted and optimized genomic DNA by molecular-genetic method RT-PCR (Real-time Polimerase Chain Reaction). The presence of genes responsible for the synthesis of antibacterial substances in genetic material, i.e., in the DNA of the bacteria *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC6633 and the microorganism *Bacillus polymyxa* isolated from the water of the Kura River, was identified by the molecular-genetic method RT-PCR.

Application of all these methods, protein-based antibacterial substances were synthesized from bacteria of the genus *Bacillus* within the framework of the scientific investigation and their presence was determined as subtilin, subtilisin and polymyxin bacteriocins.

CHAPTER III

SYNTHESIS OF ANTIBACTERIAL AGENTS BASED ON BACILLUS CEREUS ATCC 14579, BACILLUS SUBTILIS ATCC 6633 AND NATURAL BACILLUS POLYMYXA STRAINS ISOLATED FROM KURA RIVER WATER

3.1. Preparation of bacterial strains to the experiments

With purpose to revive frozen bacterial collection strains and exclude contamination, they cultured on Sheep Blood Agar and Mac-Conkey Agar. After recording their cultural characteristics and colony morphology, morphological and tinctorial indicators was examined under microscope. At the next stage, catalase, oxidase, coagulase, CAMP (Christie-Atkins-Munch-Peterson), indole tests performed, carbohydrate fermentation and H_2S secretion in TSI (Triple Sugar İron) agar investigated, motility test in tube and thin drope and a number of other manual and automatic identification tests were performed according to the rules of the ABIS system.

3.2. Study and analysis of producent characteristics of Bacillus cereus ATCC 14579 and Bacillus subtilis ATCC 6633 bacteria

The optimal growth temperatures of *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 6633 strains were determined by culturing them on Meat Peptone and Sheep Blood agars at 35; 35.5; 36; 36.5... 39.5; 40°C. Using a statistical method called variation rank, the optimal cultivation temperature of *Bacillus cereus* ATCC 14579 strain was determined to be 36.5°C, and the optimal cultivation temperature of *Bacillus subtilis* ATCC 6633 strain was determined to be 37.5°C. It was confirmed that these bacterial strains have the ability to grow rapidly under ordinary incubation conditions in inexpensive and accessible media and, therefore, can be evaluated as potential producents.

3.3. Determination of antibacterial properties of Bacillus cereus ATCC 14579 and Bacillus subtilis ATCC 6633

For investigation of the antibacterial properties of *Bacillus* bacteria against other bacteria, they were cultured side by side with the following Gram-negative and Gram-positive bacteria:

Gram-positive bacteria:
Staphylococcus aureus ATCC 29213,
Staphylococcus epidermidis ATCC 12228,
Streptococcus pyogenes ATCC 19615,
Streptococcus agalactiae ATCC 13813,
Enterococcus fecalis ATCC 29212.
Gram negative bacteria:
Escherichia coli ATCC 15922
Pseudomonas aerogenosa ATCC 27853
Salmonella typhimurium ATCC 14028
Proteus mirabilis ATCC 25933
Shigella flexneri ATCC 12022

For this purpose, the bottom of the Petri dish on Sheep Blood Agar and Meat Peptone Agar was divided into 2 semi-circles with a pencil and one side was streaked with *Bacillus* bacteria (*B. cereus* or *B. subtilis*) and the other side with other, for example, "non-*Bacillus*" bacteria. The results of the cultivation were analyzed and the antagonism of Bacillus species with other bacteria was revealed.

Alternatively, the method of testing of antagonism by Tagg and McGiven was modified and applied: the absence of growth of microorganisms around a dropes of *Bacillus subtilis* and *Bacillus cereus* onto the agar which was cultured with non-*Bacillus* suspension was observed and measured.

Antagonism of *B. cereus* ATCC 14579 against *Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus agalactiae* and *Escherichia coli*, and antibacterial action of the *B. subtilis* ATCC 6633 to the *Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus agalactiae* and *Enterococcus faecalis* were found.

3.4. Determination of the antibacterial properties of the natural B. polymyxa (Paenibacillus polymyxa) strain isolated from the water of the Kura River

For isolation of bacteria of the genus *Bacillus*, three water samples were collected with the distance of 100 meters from each other on the Kura River in the Banka settlement of the Neftchala region (geographic coordinates 39°24′50″ N, 49°14′56″ N). Each of them was prepared as a mixture of samples collected from the same point, but from a depth of 20 centimeters, 1 meter and 2 meters in sterile glass containers and mixed using a magnetic stirrer. Then, the samples (sample No. 1, sample No. 2 and sample No. 3) were diluted 1:100 with sterile distilled water and inoculated with a sterile Pasteur pipette in an amount of 0.5 ml onto Brain Heart Infusion Agar (BHIA), Muller-Hinton Agar (MHA) and Mac-Conkey agar. Mac-Conkey agar was selected to exclude Gram-negative bacteria not belonging to the genus *Bacillus*. After 24 hours of incubation at 37°C, colonies were grown in Petri dishes. Pure cultures were prepared from large single and numerous small colonies. Smears were made from gray, large, sticky, biofilm-formed colonies similar to *Bacillus bacteria* and Gram staining was performed. Short Gram-negative and long Gram-positive rod-shaped bacteria were observed during microscopy. In the next stage, pure cultures of *Bacillus* bacteria were obtained from the second and third samples of Kura water as a result of inoculation and incubation using the streaking method. In the first sample, only Escherichia coli and Klebsiella were detected. In two of the three samples (the second and third), identical *Bacillus* bacteria were detected. *Bacillus* microorganisms isolated from the Kura River formed a biofilm on agars (MHA, BHIA). This white-grayish layer completely covered the surface of the agar. Such a feature is mainly observed in *B. subtilis* and *B. polymyxa* strains. Therefore, as an additional differential test, the ability to grow in a saline environment was tested. For this purpose, a growth test was used on agar with added NaCl salt. Therefore, *B. subtilis* grows on agar with a concentration of 4% and 6% NaCl, while B. polymyxa bacteria, on the contrary, do not have the ability to grow in a concentration higher

than 2%, that is, in a saline environment. The fact that the strain isolated from the Kura water did not grow on agars with a concentration higher than 2% salt confirmed that it was *B. polymyxa*.

The impact of the Kura strain of *Bacillus polymyxa* on some Gram-positive and Gram-negative bacteria was investigated analogously to the antagonism of the *B. cereus* and *B. subtilis* collection strains. As a result of the experiments, the negative effect of the Kura strain against *Streptococcus pyogenes, Streptococcus agalactiae, Staphylococcus epidermidis, Pseudomonas aerugenos* was revealed.

In order to expose the secretion of bacteriocins to extreme, food-limited conditions, bacteria of the genus *Bacillus* were cultivated on Sheep Blood Agar for 3 days and then microscopically examined. After 24 hours of incubation at 37°C and 72 hours of incubation at 37°C, smears prepared from "young" and "old" cultures were stained by Gram and Methylene blue methods. Microscopy results were compared. In condition of food restriction, morphological signs of both bacteria changed: changes in shape, arrangement, and spore formation were observed. The phenomenon of "aging" of *Bacillus* cells was revealed. During long-term incubation, both adaptation and destruction processes result in the secretion of certain substances by bacteria to protect themselves. Therefore, three-day cultures were used for protein synthesis.

For separation of the biologically active substances, each of the *Bacillus* genus bacteria was collected in 5 ml of physiological solution in a 10 ml Falcon-type test tube. The bacterial suspensions were adjusted to an optical density of 3.0 according to the McFarland standard and placed on a conventional blood roller for shaking at room temperature at 22°C. In order to wash away biologically active substances from bacterial cells, the samples were shaken on a blood roller at a speed of 36 revolutions per minute for 18 hours. Then, before extracting biologically active substances from the solution, three biotechnological methods were tested for purifying bacterial suspensions from alive bacteria: heating in pasteurization mode, centrifugation sedimentation and bacterial filtration. As a result, the method of sedimentation of bacteria by spinning in a centrifuge at

2300 RPM for one hour at room temperature was chosen as the most effective. Complete purification of the solutions from microbes, i.e., sterility, was confirmed by the absence of any colonies when inoculating them on Sheep Blood Agar. The protein concentration in the prepared solutions was determined on a Cobas e-411 biochemical analyzer. Biologically active substances of protein origin were precipitated from the solutions with acetone and placed in a freezer for storage at a temperature of -20°C (-4°F).

CHAPTER IV

INVESTIGATION OF CHEMICAL PURITY, CONCENTRATION AND MOLECULAR MASS OF BACTERIOCINS SYNTHESIZED FROM BACILLUS CEREUS ATCC 14579, BACILLUS SUBTILIS ATCC 6633 AND NATURAL BACILLUS POLYMYXA

4.1. Sedimentation of the proteins obtained from Bacillus strains

The effectiveness of biologically active substances is directly related to their purity, that is, clean from other matters. Protein solutions obtained from *Bacillus* strains were purified by a relatively classical method - precipitation by organic solvent. In the experiments cold acetone was added at -5°C (23°F) to precipitate proteins of the *Bacillus* strains. First, 100 ml of *B. cereus* ATCC 14579 and *B. subtilis* 6633 protein solutions were collected, and 40 ml of acetone was added to both test tubes. Test tubes were placed at -5°C (23°F) freezer and checked-out every 5 minutes. After 10 minutes, the sedimentation process began in the *B.cereus* ATCC 14579 solution and was completed within 16 minutes. After 8 minutes, the sedimentation process began in the *B.subtilis* 6633 solution and was completed within 13 minutes.

4.2. Determining the purity of the synthesized protein products by the spectrophotometry in the Eppendorf Biospectrometer device

Agar-agar, which is the main component of nutrient media, contains agarose, agaropectin, D- and L-galactoses and pentose. The purity of the synthesized protein products from these carbohydrates was determined by spectrophotometry in an Eppendorf Biospectrometer. The 260/280 ratio was used to determine the purity of proteins isolated from *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* 6633 strains. The purity of the *Bacillus cereus* ATCC 14579 protein was determined to be 0.56, and the purity of the *Bacillus subtilis* 6633 protein was determined to be 0.58. Since the optimal 260/280 (carbohydrate/protein) ratio for such experiments was 0.6, the purity of the proteins was evaluated as 0.56 and 0.58 as appropriate.

4.3. Spectrophotometry of the concentration of proteins from Bacillus cereus ATCC 14579 and Bacillus subtilis 6633 strains in the NanoPhotometer device

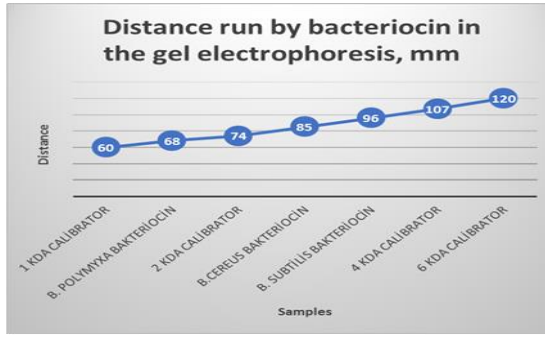
The concentration of proteins synthesized from *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 6633 strains was also determined by spectrophotometry in a NanoPhotometer device. In this experiment, proteins were measured at wavelengths of 280 nm. According to the device, the concentration of bacteriocin protein obtained from *B.cereus* ATCC 14579 is equal to 1359 ng/µl. The concentration of bacteriocin protein obtained from *B. subtilis* ATCC 6633 was equal to 1417 ng/µl.

4.4. Measurement of Molecular mass of the bacteriocins synthesized from Bacillus cereus ATCC 14579 and Bacillus subtilis ATCC 6633 strains

The concentration of bacteriocin obtained from the natural *B. polymyxa* strain isolated from Kura water was determined to be

1547 ng/ml. The protein concentration obtained from the natural strain slightly higher than gotten from *B. cereus* ATCC 14579 (1357 ng/mml) and *B. subtilis* ATCC 6633 (1417 ng/mml). The above-mentioned experiments have shown that the *B. polymyxa* bacterium isolated from the nature of Azerbaijan, especially from the water of the Kura River, is a productive and efficient producent. Measurement of the Molecular mass (Mm) of bacteriocins was conducted in vertical electrophoresis. A graph was prepared with the molecular weight of known proteins (calibrators) and the distance passed in the gel, and the molecular weight of unknown proteins was determined on the graph. The logarithmic correlation between molecular weight and the distance traveled by proteins in the electric field is reflected in the graph (Graph 4.1) and the table (Table 4.1). Protein standards with molecular masses of 1, 2, 4 and 6 kDa were used to perform the calculation. Based on the graph, the molecular mass of the protein obtained from the *Bacillus cereus* strain (BC) was 3.6 kDa, from *Bacillus* subtilis (BS) 26 kDa, and from *Bacillus polymyxa* 1.6 kDa.

It is known that the molecular mass of subtilin is approximately 3.6 kDa, the molecular mass of subtilisin is 26-27 kDa, and the molecular mass of polymyxin is 1.4-1.6 kDa, and it is assumed that the synthesized proteins are subtilin, subtilisin, and polymyxin. The above experiments have shown that the natural *Bacillus polymyxa* from Azerbaijan is a productive and efficient producer.



Graph 4.1. Measurement of molecular mass of new bacteriocins

Table 4.1

Distance run by bacteriocins in the gel electrophoresis

Bakteriocins and calibrators	Distance run by bacteriocin in the gel electrophoresis, mm
1 kDa calibrator	60
2 kDa calibrator	74
Bacillus cereus bakteriocin	85
Bacillus subtilis bakteriocin (1:10 dilution)	96
Bacillus polymyxa bakteriocin	68
4 kDa calibrator	107
6 kDa calibrator	120

4.5. Spectrophotometric measurement of the concentration and purity of bacteriocin obtained from the natural Bacillus polymyxa strain isolated from Kura water

Spectrophotometric measurement of the concentration and purity of bacteriocin obtained from the natural *Bacillus polymyxa* strain isolated from Kura water was carried out analogously to the experiments conducted on bacteriocins of collection strains. The concentration of bacteriocin obtained from the natural *Bacillus polymyxa* strain isolated from Kura water was determined to be 1547 ng/ml, and its chemical purity was 0.59. The above experiments showed that *B. polymyxa* bacteria isolated from the nature of Azerbaijan, from the water of the Kura River, are productive and efficient producers.

Usually, bacteria of the genus *Bacillus* are isolated from the soil, but Kura water is a turbid, silty and, in fact, it is a substrate mixed with soil, and microorganisms at its bottom are also found in Kura water. In this regard, it is easy and convenient to isolate bacteria of the genus *Bacillus*, especially *B. polymyxa*, from Kura water. Its ability to secrete numerous antibacterial substances and its indicator bacteriocin concentration are almost equal to the same concentration of the

collection strains. Thus, during the experiments, it was found that the studied production properties of *B. polymyxa* are identical to the production properties of the collection strains.

CHAPTER V

INVESTIGATION OF ANTIBACTERIAL PROPERTIES OF BACTERIOCINS SYNTHESIZED FROM BACILLUS CEREUS ATCC 14579, BACILLUS SUBTILIS ATCC 6633 AND BACILLUS POLYMYXA ISOLATED FROM KURA WATER

In the framework of the study, protein substances were obtained from bacterial strains *B. cereus* ATCC 14579 and *B. subtilis* ATCC 6633. For investigation of their antibacterial properties, experiments were conducted in two directions:

1. Synthesised bacteriocins were applied to nutrient media inoculated with bacteria in Petri dish in the form of drops and the growth of Gram-positive and Gram-negative bacteria was observed.

2. According to the Tagg[24] and McGiven method, agar was soaked in Petri dishes with a bacteriocin solution (0.5 ml for each dish). After spreading the solution on agar, five Gram-negative and five Gram-positive bacteria were inoculated onto the Petri dishes with agar and, as a negative control, pure agar was used. It is clear from the graph that the new bacteriocins significantly inhibit the growth of bacteria *St. aureus, St. epidermidis, Str. pyogenes, E. fecalis* and *E. coli.* This bacteriostatic effect is reflected in the following diagram and table. (Figure 5.1) (Table 5.1)

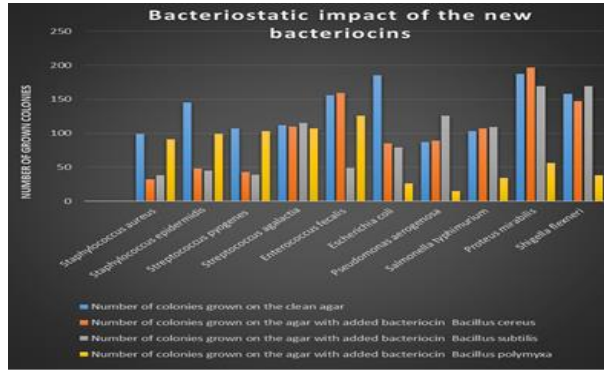


Figure 5.1. The inhibition effect of the synthesized bacteriocins to the growth of certain bacteria

Usually, two indicators are calculated to determine the bacteriostatic effect of antibacterial substance: the Minimum Inhibitory Concentration indicators MIC_{90} or MIC_{50}. MIC_{90} and MIC_{50} values is the lowest concentration of the antibiotic at which 90 and 50% of the isolates were inhibited, respectively. There are few ways of measuring the MIC, including disc diffusion testing according to Kirby-Bauer, E-test using gradient stripes and micro-dilution, which is usually performed in automates like Vitek, Phoenix or WalkAway. In this investigation MIC_{50} was identified by calculation. In these experiments, the concentrations of bacteriocins were known. The number of inhibited colonies is equal to the difference between the number of colonies on pure agar and on agar supplemented with bacteriocin. 50% inhibition of colonies was determined by calculation.

The Minimum Inhibitory Concentration of Bacteriocins MIC_{50} was calculated using the proportional algebra method. For example, 99 colonies of *St. aureus* were grown on pure agar. 50% of 99 colonies is equal to 45.5 colonies. Bacteriocin obtained from *Bacillus cereus* at a concentration of 1357 ng/µl killed 32 colonies, and 67 colonies were not killed. We construct the proportion:

1357 ng/µl concentration - 67, i.e. 99 minus 32 colonies inhibits
X ng/µl MIC_{50} - 45.5 colonies, i.e. 50% of 99 colonies inhibits.

X = 1357 x 45.5 / 67 = 921.5 ng/µl

That is, the MIC_{50} of the bacteriocin obtained from *Bacillus cereus* ATCC 14579 against *Staphylococcus aureus* ATCC 29213 strain is 921.5 ng/µl. Analogously, the MIC_{50} of the other three bacteriocins was calculated (Table 5.2).

Table 5.1

Growth of Gram-positive and Gram-negative bacteria on agar soaked with synthesized bacteriocins

№	Name of bacteria	Number of bacteria grown on pure agar	Number of bacteria grown on bacteriosin added agar		
			B. cereus ATCC 14579	B. subtilis ATCC 6633	B. polymyxa
1	St.aureus ATCC 29213	99	32	38	91
2	St.epidermidis ATCC 12228	145	48	45	99
3	Str.pyogenes ATCC 19615	107	43	39	103
4	Str.agalactiae ATCC 13813	112	110	115	107
5	E. fecalis ATCC 29212	156	159	49	126
6	Escherichia coli ATCC 15922	185	85	79	26
7	Ps.aerugenosa ATCC 27853	87	89	126	15
8	S.typhimurium ATCC 14028	103	107	109	34
9	P. mirabilis ATCC 25933	187	197	169	56
10	Sh. flexneri ATCC 12022	158	147	169	38

Table 5.2

MİC$_{50}$ of the new bacteriocins

№	Name of bacteria	Number of bacteria grown on pure agar	MİC$_{50}$		
			B. cereus ATCC 14579 1357 ng/mkl	B. subtilis ATCC 6633 1417 ng/mkl	B. polymyxa 1547 ng/mkl protein
1	St.aureus ATCC 29213	99	901	1033	not
2	St.epidermidis ATCC 12228	145	1014	1027	2438
3	Str.pyogenes ATCC 19615	107	1134	1115	not
4	Str.agalactiae ATCC 13813	112	not	not	not
5	E. fecalis ATCC 29212	156	not	1032	4022
6	Escherichia coli ATCC	185	1255	1236	900
7	Ps.aerugenosa ATCC 27853	87	not	not	934
8	S.typhimurium ATCC 14028	103	not	not	804
9	P.mirabilis ATCC 25933	187	not	not	1104
10	Sh.flexneri ATCC 12022	158	not	not	1018

CHAPTER VI

GENETIC EXAMINATION OF B. CEREUS ATCC 14579, B. SUBTILIS ATCC 6633 AND NATURAL BACILLUS POLYMYXA STRAINS

6.1. Identification of novel protein-derived antibacterial substances

Within the framework of genetic examination of *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6633 and natural *Bacillus poymyxa* strains, the genetic basis of the natural antibiotics subtilin, subtilisin and polymyxin was investigated. The selection of these biocins was due to the fact that they have the same molecular mass as the synthesized biocins. The PCR method was used in the following experiments.

6.2. DNA extraction, optimization and DNA measurement of the Bacillus isolates

DNA was extracted from *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 6633 isolates, measured and the genetic basis of the subtilin and subtilisin proteins, i.e. the presence of the sboA and spaS genes, in its composition was checked by PCR. Since the concentrations of the samples were 130 and 118 ng/ml, both were dissolved, the concentration was optimized and measured again. When measured again with a spectrophotometer, the DNA of the Bacillus cereus ATCC 14579 culture was 36 ng/ml and the purity was A260/A280 1.7. The DNA content of the *Bacillus subtilis* ATCC 6633 sample was 41 ng/ml (optimal concentration 20-60 ng/ml) and the purity was A260/A280 1.8 (optimal purity < 2).

6.3. Determination of the genes of subtilin, subtilisin and polymyxin bacteriocins by PCR analysis of Bacillus cereus ATCC 14579, Bacillus subtilis ATCC 6633 and Bacillus polymyxa DNA samples isolated from Kura River.

After the bacterial DNA was extracted, PCR reagents were prepared. Specific primers were used for the *sboA* and *spaS* genes responsible for the synthesis of subtilin and subtilisin.
Forward and reverse primers of the Subtilisin gene belonging to *B. subtilis* bacteria:
Forward:
sboA-f (5′-CATCCTCGATCACAGACTTCACATG-3′)
Reverse:
sboA-r (5′-CGCGCAAGTAGTCGATTTCTAACAC-3′)

Primers for determining the Subtilin gene belonging to *B. subtilis* bacteria:
Forward:
spaS-f (5′-TGTCATGGTTACAGGCGGTATCGGTC-3′)
Reverse:
spaS-r (5′-AGTGCAAGGAGTCAGAGCAAGGTGA-3′)

Invitrogen reagents were chosen to carry out the reaction. PCR was performed on a Biorad CFX-96 PCR device. During the study, determination of *sboA* and *spaS* genes was carried out at the same time. Amplification program was written to the thermocycler of the Biorad device:

1. Thermocycler heating 95°C - 15 minutes
2. Denaturation 0.05 minutes at 95°C
3. Annealing 0.20 minutes at 60 °C
4. Elongation 0.15 minutes at 72 °C
5. 5 cycles starting from step 3
6. Denaturation 0.05 minutes at 95°C
7. Annealing 20 seconds at 60 °C
8. DNA - copy counting - Reading
9. Elongation 0.15 minutes at 72 °C
10. 40 cycles starting from step 7

Analogously to the first PCR reaction, a Real-Time PCR reaction was performed on the Biorad CFX-97 instrument using these

primers and the polymyxin pmxE gene was detected in the DNA of the *Bacillus polymyxa*. The results of the genetic examination showed that the genetic basis of the polymyxin bacteriocin, i.e. the pmxE gene, is present in the *Bacillus polymyxa* strain (intersection with the "threshold" 25.82 cycles). The above-mentioned genetic experiments and molecular mass measurements showed that antibacterial substances of protein origin were synthesized: subtilin from the *Bacillus cereus* ATCC 14579 strain, subtilisin from the *Bacillus subtilis* ATCC 6633 strain, and polymyxin from the natural *Bacillus polymyxa* strain. The device reports (original protocols) and quantitative results of the polymerase chain reactions conducted on the "Biorad" CFX-96 device to determine the genes responsible for bacteriocin synthesis were analyzed.

FINAL ANALYSIS OF THE RESULTS OBTAINED IN THE RESEARCH

Due to the microbial resistance caused by antibiotics and a number of their shortcomings, there is a need to search for new high-quality medicines. In this regard, antibacterial substances of natural biological origin, that is, bacteriocins, have become the focus of special attention of scientists. In addition, the application of bacteriocins in various fields makes research in this direction of science more promising. The discovery of new bacteriocin-producing strains is a very important issue for medicine, veterinary medicine, agriculture, food industry, farming, horticulture and other fields. Studying the properties of bacteriocins, selecting bacteriological environments for producer bacteria, determining optimal conditions and adapting synthesis methods to industrial conditions is very important for modern biotechnology. In general, various methods for obtaining new bacteriocins have been tested and successfully implemented in the scientific world. During the four-year research conducted in the laboratory, new variants of useful methodologies for laboratory practice and scientific research have been proposed. For example, so far, more than a hundred different antibacterial substances have been prepared and put into use based on bacteria of

the genus Bacillus. However, most of these substances exist only theoretically. In practice, their synthesis is complex and requires serious financial and special conditions for implementation. Taking all this into account, the task of selecting and finding more suitable, more efficient and convenient methods was fulfilled during the research. Additional antibacterial properties of *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 14579 and natural *Bacillus polymyxa* microbes, which have not been mentioned in the scientific literature until now, were discovered. The experiments described in this scientific work can be applied for the mass production of various bacteriocins on an industrial scale. The described experiments can help to extract biologically active substances not only from bacilli, but also from suspensions of other bacteria.

During the detailed theoretical studies and laboratory experiments carried out within the framework of the scientific work, in accordance with the tasks set for the research, the main results were obtained that increase the knowledge of biotechnology science and can be applied in the future.

RESULTS

1. The productivity and antibacterial properties of the selected strains of the genus *Bacillus* were studied. It has been found that *Bacillus cereus* ATCC 14579 has a bacteriostatic effect against *Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus agalactiae and Escherichia coli. Bacillus subtilis* ATCC 6633 has a bacteriostatic effect against *Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus agalactiae, Enterococcus fecalis*. The natural *Bacillus polymyxa* strain isolated from the Kura River has a bacteriostatic effect against *Streptococcus pyogenes, Streptococcus agalactiae* and *Staphylococcus epidermidis* [1, 2, 4, 6, 10].

2. Protein-based antibacterial substances (subtilin, subtilisin and polymyxin) have been synthesized from bacteria of the genus *Bacillus* (from the collection strains *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 6633) and from the natural *Bacillus polymyxa* strain

isolated from the Kura River) [7, 9, 11, 14].

3. The chemical purity, concentration, and molecular weight of bacteriocins synthesized from selected *Bacillus* strains were measured, and their antibacterial properties were determined[14, 17].

4. The genetic basis of subtilin, subtilisin, and polymyxin bacteriocins was confirmed in the genetic material of the *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 6633 collection strains and the natural *Bacillus polymyxa* strain isolated from the Kura River: the subtilin spaS gene was detected in the *Bacillus subtilis* ATCC 6633 DNA, the subtilisin sboA gene was detected in the *Bacillus cereus* ATCC 14579 DNA, and the pmxE gene was detected in the *Bacillus polymyxa* DNA[3].

5. In addition to the objectives of the study, the "aging" process of *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 14579 bacteria was detected after three days of incubation, in this regard changes in their morphology and location were described [11].

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The defense will be held on January,15 2025 at 11:00 AM at the meeting of the Dissertation Council BFD 1.07/1 of Supreme Attestation Commission under the President of the Republic of Azerbaijan operating at the Institute of Microbiology,the Ministry of Science and Education of the Republic of Azerbaijan.
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Electronic versions of the dissertation and its abstract are available on the official website of the Institute of Microbiology, the Ministry of Science and Education of the Republic of Azerbaijan (https://azmbi.az/index.php/az/).
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