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Trials for Synthesis of Bact Eriocins from the Bacteria Genus *Bacillus*

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Abstract

In the presented article, experiments for determination of antibacterial properties of the strains *Bacillus Cereus* ATCC 14579 and *Bacillus Subtilis* ATCC 6633 and, therefore, their ability to secrete bacteriocins, was subscribed. There are many trials performed for choosing the most affordable, the least time-consumable and the most appropriate method for obtaining bacteriocins from those strains. Possibility of adaptation, the synthesis way to conditions of a factory production was analysed. During that, research productivities of processes such as filtration, pasteurisation, and scrolling in a centrifuge of the bacterial suspensions were comprised. Usage of supernatant, obtained after long-lasting centrifugation of the bacterial suspensions, prepared after 72 incubation of the bacteria was received as most suitable for obtaining of the biologically active metabolites. Additionally, during the experiments, incidentally, was discovered, that both *Bacillus* bacteria strains from fresh 24 hours culture and old 72 hours culture isolates differ in morphology signs. This may, generally, contributes to the emergence of a new approach to the study of the morphology of other "young" and "old" bacteria.

Keywords: bacteriocin, *Bacillus cereus*, *Bacillus subtilis*, syntheses, antibiotic, biotechnology

Introduction

The problem of resistance of microorganisms to antibiotics is quite urgent. Using of antibiotics leads to the development of multi-resistant forms of microbes, causes hospital infections, has a polluting effect to the environment, destroys the microbiota of biocenoses, suppress the immune system and has a number of side effects. One of the options for solving this challenge is the development and introduction of natural antibiotics of bacterial origin - bacteriocins. Various methods of obtaining them have

been tested and successfully implemented. In particular, more than 100 different antibacterial agents were obtained on the basis of bacteria of the genus *Bacillus*. However, in practice, their synthesis is rather complicated and requires serious financial investments and special equipment. The history of bacteriocin substances goes back about a hundred years. The first bacteriocin named "colicin V" was discovered in 1925 by the scientist Andre Gratia at the Pasteur Institute in Paris. Since then, more than 100 such antibacterial agents have been isolated and synthesized. For example, the BACTIBASE database contains information on 123 bacteriocins, which are metabolites of Gram-positive and Gram-negative bacteria. (Hammami *et al.,* 2007) Structurally, bacteriocin is synthesized on ribosome low molecular weight hydrophobic protein or peptide toxin produced by bacteria with the purpose to inhibit the growth of the similar or closely related concurrent bacterial strains. When exposed to a hostile environment or nutrient restriction, these substances help bacteria survive and give them an edge over other closely related species. These substances are highly effective at low pico- and nanomolar concentrations and are specific for certain types of bacteria. (Zaslavskya *et al.,* 2019) Despite the fact that these substances are not inferior in antibacterial activity, and sometimes even surpass classical antibiotics and do not cause resistance in bacteria (Svetoch *et al.,* 2011), their usage now is limited mainly to the food industry, veterinary medicine, and agronomy. Only a few bacteriocins, such as baneocin, polymyxin, gramicidin and bacitracin, were implemented to medicine for the treatment of various infections. Meanwhile, it was proven that bacteriocins also have antiviral, antifungal and antitumor effects. They stimulate the body's immune responses, induce immunity, and this safe products of the vital activity of bacteria are usually found in meat and dairy foods. In connection with the above, the development and introduction of new bacteriocin drugs into pharmacology is a promising and urgent direction of modern biotechnology. (Awais *et al.,* 2010)

The purpose of our study was developing of methods for the extraction of bacteriocins from bacteria of the genus Bacillus, or rather two strains of this genus - *Bacillus subtilis* ATCC6633 and *Bacillus cereus* ATCC 14579. The fact is that both species *Bacillus subtilis* and *Bacillus cereus* are active producers of bacteriocins. (Taghiyeva, 2019; Taghiyeva, 2020). Even throw the antibacterial properties of these particular strains have not yet been investigated. We set the task to find the most affordable, convenient and reproducible in production conditions for obtaining bacteriocin preparations based on the above strains.

During the review of the scientific literature, it was found that scientists, usually/ generally, uses/applys the methods of microbiology, biotechnology, and genetic engineering to synthesize the bacteriocins, for instance, in the work of American scientists (Laura *et al.,* 1994).

The article by Brazilian scientists describes the production of the collagenase enzyme from *Bacillus cereus*. The enzyme was isolated and its molecular weight was measured using a zymogram technology of electrophoresis on polyacryl gel. (Pequeno & Arruda, 2019)

When another enzyme proteinase was extracted from *Bacillus Cereus*, the proteinase activity was studied by cleavage of gelatin. Holes were made in agar containing gelatin, a sterile filtrate of 12 hours culture was poured into them and indicated. After that the diameter of the lysis-proteolysis zone around the wells was measured after 12 hours. Galactose and peptone were chosen as a source of carbon and nitrogen during the experiment. The second method consisted of additional 4% inoculums to nutrient broth with a special composition. Then the flask was rocked, centrifuged and the supernatant was used to purify the mixture, repeated centrifugation was used to pass it through filters at low temperatures, then the protease was precipitated by ammonium sulfate. At each stage, measurements were carried out using spectrophotometer. (Gaurav & Prakash, 2015)

Many of these methods, as a rule, are expensive and time consuming. They require special high-tech equipment, highly qualified personnel, as well as restrictions of a microbiology laboratory Class II. At the same time, the yield of the drug is very low and is completely unsuitable for factory. For use on an industrial scale, technology is required that does not need high costs and special conditions. Therefore, it was necessary, based on the experience accumulated by scientists, to develop new methods optimized and adapted, taking into account the cost and time spent to syntheses of the new bacteriocin substances.

Material and methods

For study of the antibacterial properties of the *Bacillus subtilis* ATCC6633 and the *Bacillus cereus* ATCC 14579 strains, we have developed special methods, based on co-cultivation of these strains with Gram-positive bacteria *Staphylococcus Aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 122286), *Streptococcus aqalactia* (ATCC 13813), *Streptococcus pneumonia* (ATCC 49619), *Streptococcus pyogenes* (ATCC 19615) and Gram negative bacteria *Escherichia coli* (ATCC 15922), *Pseudomonos aerogenosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Proteus mirabilis* (ATCC 25933). It was found that *Bacillus subtilis* ATCC6633 inhibited growth of these bacterial strains: *Staphylococcus epidermidis*, *Streptococcus pyogenes*, and *Streptococcus agalactia*, while *Bacillus cereus* ATCC 14579 inhibited the growth of the corresponding bacterial strains of *Staphylococcus*

epidermidis, *Streptococcus pyogenes*, *Streptococcus agalactia*, *Escherichia coli* and *Pseudomonos aeruginosa.*

To obtain a new bacteriocin, especially freeing of bacterial suspension from bacterial cells, various methods were tested: centrifugation, filtration, precipitation, and others. More than 1000 experiments have been carried out. It was assumed that the concentration of antimicrobial drug would be significantly higher, if bacteria of the genus Bacillus is placed in conditions of lack of nutrients. Therefore, these two strains were incubated on Sheep Blood Agar for 3 days at 37° C. During the microscopy of that isolates, based on smears with methylene blue and Gram stain, a change in the morphology of bacteria was observed. The bacteria of a fresh pure culture after a four-day incubation changed both the shape and size of the vegetative and spore cells. Such processes confirmed the presence of adaptation and destruction of cells and supposed the release of certain toxins from the bacteria. (See Table 1.) It was observed interesting fact of aging of the bacterial, particularly Bacillus cells. By our opinion, this theory has to change an approach to investigate bacterial morphology.

Table 1. Comparison of morphological signs of the bacterial cells *Bacillus subtilis ATCC6633* and *Bacillus cereus ATCC 14579* after 24 and 72 hours of incubatin on Sheep blood aqar.

No	Indicators	*Bacillus cereus ATCC 14579*		*Bacillus subtilis ATCC6633*	
		12 hours incubation	72 hours incubation	12 hours incubation	72 hours incubation
Vegetative form					
1.	Number of typical bac-teria in smear	99%	abut 30%	99%	about 40%
2.	Form of bacteria	Bacilli	Bacilli and cocco-bacilli	bacilli	Cocco-bacilli and prolongated swirling (snail-shaped) bacilli
3.	Size	Normal	smoller	normal	Small and longer
Spores					
4.	Size	small, about 1/6 bacilli size	Large, about1/3 bacilli size	small, about 1/6 bacilli size	Large, about1/3 bacilli size
5.	Number of spores	Few	in 50% of bacilli	few	in 60% of bacilli

After incubation for the extraction of biologically active substances, the bacteria of the genus *Bacillus* of both strains were collected to sterile tubes with saline and was

sent to a regular blood roller for washing-out of the bacterial cells and release of biologically active matters. After rocking for 18 hours, experiments were carried out to free the suspension from bacteria using three methods: pasteurization, collecting the supernatant after centrifugation, as well as filtering in various modes. It is known that classic pasteurization is a process of heating of the milk products at 63 °C (145 °F) for 30 minutes. By new pasteurization mood, it is quick heating to 72 °C (162 °F) for 15 seconds. It was concerned that convenient yield of protein in the suspension will be more stable at 63 °C (145 °F), but after such attenuation some amount of bacterial cells stayed alive and able to multiply at subsequently streaking to agar. Pasteurization mood 72 °C (162 °F) for 15 was more successful in relation to freeing from bacteria, but such heating leads to denaturation and deactivation of protein-linked substances. In conclusion, it was received that pasteurization method is not suitable for extraction of biologically active substances from bacterial suspension. Otherwise, the most successful method for freeing from the bacterial mass turned out to be the centrifugation method: the tubes were spun for an hour at a speed of 2300 rpm and then the carefully taken supernatant was inoculated on blood agar to confirm sterility. New antibacterial substances were extracted by precipitation with ammonium sulfate. Protein amount of the supernatant was checked-out by biochemistry analyzer Cobas e111 and contained from 0.1g/l to 0.5 g/l. The precipitated proteins were collected by centrifugation for 30 minutes at 5000 rpm and dissolved in Tris buffer.

Subsequent experiments show that the antimicrobial substances synthesized in this way had the same inhibitory properties as the culture of bacterial cells of the selected strains.

Results and discussion

Although we are familiar with many high technologies, in experiments we tried to find out easy and affordable method useful for production of bacteriocine in future. Such simple methods as centrifugation and precipitation were used for extraction of antibacterial substance from broth.

As a result of the research, the antibacterial properties of the strains *Bacillus subtilis* ATCC6633 and *Bacillus cereus* ATCC 14579 were confirmed and new peptides were synthesized. These proteins inhibited the growth of mainly Gram-positive bacteria - staphylococci and streptococci. It was observed that from the methods, selected for cleaning of bacteriocin solution from alive bacterial cells (filtration, pasteurization and centrifuge sedimentation), the most suitable method is centrifuge sedimentation.

Accidentally, it was observed one very rare described phenomenon of aging of the bacteria and morphological differentiation of *Bacillus* bacteria in fresh and old culture. Continues scientific work to identify the synthesized substances and study their physicochemical, biological and antibiotic indicators was expected. Scientific investigation in this direction is to be continued and the results are quite promising for intended purpose.

Conclusion

Developing of technological methods for syntheses of natural antibacterial bacteriocins is very important for microbiology and biotechnology sciences. This research will allow providing medicine, veterinary and agriculture with safe, effective and affordable antibacterial substances in alternative to classic antibiotics. Usage of bacteria of *Bacillus* genus opens wide perspectives for this type of research.

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