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Determination of Antibacterial Properties of the Bacillus Cereus ATCC 14579 Strain for Application in Synthesis of Bacteriocins by New Methods

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Abstract

Nowadays bacteriocins occupy an important niche among the wide range of various antimicrobial matter, including synthetic antibiotics, pesticides, disinfectants, bacteriofages, and so on. Sources of those substances are different bacteria capable of producing such matter for self-protection against bacteria in conditions connected with limitations of nutrients and inappropriate environmental changes. Bacteriocins are characterized as safe for human health and the welfare of animals; they do not cause side effects and resistance by microbes like ordinary antibiotics. That is why scientific investigations into the development of new bacteriocins, as well as the implementation of new antibacterial substances in medicine, veterinary treatment, food production, plant sanitary are necessary. But the number of bacteria really suitable for such syntheses is very small. Bacillus cereus and Bacillus subtilis bacteria are most convenient for such process. Thus, this study was dedicated to investigating the antimicrobial properties of the bacterium Bacillus cereus strain ATCC 14579 as a possible producer of new bacteriocins. Especially for this investigation the author developed two original methods. The aforementioned strain was opposed to different Gram-positive and Gram-negative bacteria in laboratory conditions, and a certain antagonism was found. In particular, in experiments Bacillus cereus inhibited the growth of such Gr+ bacteria as Staphylococcus Epidermidis, Streptococcus pyogenes and Streptococcus agalactiae, as well as Gr- bacteria Escherichia coli and Pseudomonas aeruginosa. Subsequenced research should be continued to obtain antimicrobial agents from ATCC 14579.

Keywords: bacteriocins; *Bacillus cereus*; antagonism of bacteria; new method

Introduction

The Bacillus cereus bacterium combines more than 100 various strains. Some of the strains are harmful to humans and animals and able to cause toxic infection - food poisons - but others are harmless saprophytes. This bacterium is an intestine and food inhabitant that can active multiply in meals like vegetables, eggs, meat and dairy products and is capable of producing, in food and livestock feed, two types of toxins: emetic toxin and enterotoxin **(**Turnbull *et al.*,1983) Immunocompromised people and animals are more susceptible to Bacillus cereus bacteremia, and the endocarditis, meningitis, pneumonia and endophthalmitis caused by this bacterium. Conversely, other strains of these bacteria are known as sources of probiotics that activate growth and development in cattle and sheep (Charalampopoulos *et al*, 2009). They can also provocate abortion in cattle, related to placental necroses (Schuh & Weinstock, 1989) During intestinal colonization the opportunistic human pathogen *Bacillus cereus* demonstrates antimicrobial activity against concurrent Gram-positive and Gram-negative bacteria by excreting antimicrobial matter. For these properties, Bacillus cereus may be used for the production of natural antibacterial substances (bacteriocins). Generally, bacteria of the genus Bacillus justified themselves as producers of bacteriocins and other biologically active substances, not only on a scientific, but also on an industrial scale. Moreover, they are quite accessible, since they are widely distributed in the environment: water, soil, dust and air, and are easily cultivated on ordinary nutrient media, requiring no special conditions for reproduction, and are stable. For example, Bacillus cereus bacteria can multiply within a wide temperature range from 4 ° C (39.2 ° F) to 55 ° C 131 ° F and within acidity of pH 5.3 to 9.3. As potential producers, being facultative aerobes, they do not require special conditions for reproduction, and due to their spore formation, they are well preserved in the environment. (Duaa, 2005).

The metabolism of Bacillus cereus strains has been studied. For example, in a study by American scientists, “Biological Activity of Two Fungiostatic Antibiotics Isolated from Bacillus cereus UW85,” antifungal activity was observed in the form of inhibition of budding of the herm-tube and in Petri dishes by diffusion on nutrient agar. Then isolated eluate was tested for antifungal activity. In addition, Bacillus cereus UW85 was mutated and the mutant strains’ ability to inhibit the growth of fungi was tested, too (Laura *et al.,* 1994).

Practical applications of particularly strain ATCC 14579 were completed with the production of restriction enzyme endonuclease Bce14579 food testing (as a control strain for bacteriological tests) (https://lifescience.invitro.com.au/products /s/ATC14579/Bacillus-cereus/).

Publications describing the antibacterial metabolites of B. cereus ATCC 14579 also known as the Frankland and Frankland strain are extremely limited. Only one Norway study reports that scientists obtained a bacteriocin-like inhibitory substance (BLIS) with molecular mass 3.4 kDa. (Risøen, 2004)

That is why our scientific investigations were dedicated to confirming the ability of particularly the strains that inhibit growth of other bacteria in laboratory conditions, for a determination of ATCC 14579 as an appropriate strain for syntheses of bacteriocins. A determination of the antibacterial properties of the bacillus cereus ATCC 14579 strain for application in the synthesis of bacteriocins by new methods were conducted.

Material and Methods

For the detection of bacterial antagonism, two original methods were developed by the author. For the first method (See Figure 1), during the experiments Bacillus cereus ATCC 14579 was co-cultured with five Gram-positive and five Gram-negative bacteria: Bacillus cereus on the "upper" semicircle, the other bacteria on the "lower" semicircle of the Petri dish. The second method (See Figure 2) was similar to the identification of bacteria using a bacteriophage, dropped onto bacterial culture, but in our method Bacilus cereus and Bacillus subtilis inoculates were added as two different drops to agar, freshly cultured and dried for a few minutes, and not to ready colonies as in the bacteriophage typing method. Biosafety and biosecurity rules were strictly followed, as well as good laboratory practice (World Health Organisation) and principles too.

For the purity of the experiment, lyophilized ATCC (American Type Culture Collection) strains delivered from USAMRİİD (The United States Army Medical Research Institute of Infectious Diseases) were customized. No wild strain was obtained from the environment. From lyophilized bacteria suspensions in Soybean Broth, supplemented with 10% glycerol to McFarland standard 4.0, were prepared, aliquoted and frozen. The first part of the experiments proved the viability of the bacterial strains and their suitability for laboratory studies. Bacillus cereus (ATCC 14579), Bacillus subtilis (ATCC 6633), Gram-positive bacteria Staphylococcus aureus (ATCC 29213), Staphylococcus epidermidis ATCC 12228), Streptococcus pyogenes (ATCC 19615), Streptococcus aqalactiae (ATCC 13813), Enterococcus faecalis (ATCC 29212) and Gram-negative bacteria Escherichia coli (ATCC 15922), Pseudomonas aeruginosa (ATCC 27853), Salmonella typhimurium ATCC 14028), Proteus mirabilis (ATCC 25933), Shigella flexneri (ATCC 12022) were

cultured and tested. https://www.atcc.org/en/Products/Cells_and_Microorganisms/Bacteria.aspx

Figure 1. First original method for the determination of bacterial antagonism between Bacillus cereus and Enterococcus faecalis. Bacillus Cereus colonies covered the whole plate, but Enterotococcus colonies were not visible at all.

To verify the suitability of the cultured bacteria, they were re-identified for confirmation of bacterial strain and pure culture by colony morphology, microscopy, biochemical and other additional test. For supporting manual tests, strains were also identified on the Vitek® 2 Compact 30 (French bioMerieux) instrument. Here, suspensions of bacterial cultures were added to disposable cartridges and the machine tested the cultures over 5 hours. The results of the manual and automatic identifications confirmed the suitability of the bacteria for the study.

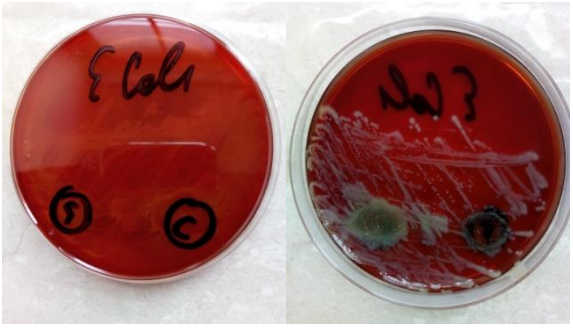

Figure 2. Second original method for determination of bacterial antagonism between Bacillus cereus (C) and Bacillus subtilis (S). Look at the zone under and around the dropped area. Here Bacillus Cereus have grown under and far around the drop. In the left figure - outer side, on the right figure - inner side of the same plate.

In the main course of the laboratory experiment for identification of the antibacterial properties of Bacillus Cereus, all bacteria were cultured on Meat Peptone Agar and Blood agar (with the addition of sheep red blood cells) and tested. As well as bacterial cultures, culture media were pre-tested, that is, their sterility and ability to provide normal bacterial growth were confirmed. In the first method, each Petri dish was divided on the outer lower side by a marker into two equal semicircles and labelled with the names of the bacteria. On each plate, Bacillus Cereus was streaked onto one semicircle and one of the Gram-positive or Gram-negative bacteria onto the other semicircle. The experiment was carried out repeatedly using both nutrient media. At the same time, all biosafety rules were followed to prevent contamination and false results. After cultivation at 37 ° C for 18 hours, the joint growth of bacteria was analysed. Inhibited growth (rare or absence of colonies) of some bacteria near the line of intersection was recorded. In the second method Bacillus cereus and Bacillus subtilis inoculates (suspensions) were added as two different drops to agar, freshly cultured and dried for a few minutes, then incubated at 37 ° C for 18 hours and a “clear zone” around the Bacillus of other bacterial colonies was detected. Most of the Petri dishes were photographed and images systematized. As a result of both methods it was found that bacteria of the genus Bacillus, especially Bacillus cereus, actually demonstrated antibacterial properties in vitro. That means that Bacillus cereus ATCC 14579 slows down, and sometimes completely inhibits, the growth of other bacteria. The outcomes of the experiments are shown in Table 1. The text describing the character of antagonism detected is shown in italics.

Table 1. Analysis of joint bacteriological culture of bacteria of the genus Bacillus with other bacteria in order to detect antagonism.

No	Bacterial name	*Bacillus Cereus*
Gram-positive bacteria		
1	*Staphylococcus aureus*	Antagonism not observed
2	*Staphylococcus epidermidis*	On both the Blood and Meat Peptone agar, Bacillus cereus interferes with the normal growth of Staphilococcus Epidermidis
3	*Streptococcus pyogenes*	On the Meat Peptone agar, Bacillus cereus interferes with the normal growth of Streptococcus pyogenes and some colonies of B.C. growing on the side of Str.P. On Blood agar near B.C. Str.P colonies are very rare
4	*Streptococcus aqalactiae*	On Meat Peptone agar, Bacillus cereus interferes with the normal growth of Streptococcus agalactiae and some colonies of B.C. growing on the side of Str.A. On Blood agar, Str.A colonies are very rare and small
5	*Enterococcus faecalis*	Antagonism not observed

Qram-negative bacteria		
6	*Escherichia coli*	Bacillus cereus interferes with the normal growth of E. Coli, part of the colonies of Bacillus Cereus grow on the side of E.C.
7	*Pseudomonas aerogenosa*	Bacillus cereus inhibits normal growth of Ps.A.
8	*Salmonella typhimurium*	Antagonism not observed
9	*Proteus mirabilis*	Antagonism not observed
10	*Shigella flexneri*	Antagonism not observed

Results and discussion

Thus, the bacteria Bacillus Cereus were repeatedly plated on Blood and Meat-Peptone agars in pairs together with one of ten bacteria. During the experiments, it was proved that these bacteria actually have the property of inhibiting the growth of other bacteria by some biologically active substances secreted by them into the nutrient medium. Moreover, in some cases, they completely or partially suppressed the growth of colonies of another bacterium; in other cases, Bacillus colonies “crossed” the conditional border intersection and grew on the semicircle where the non-Bacillus bacteria were cultured. Sometimes between the colonies of Bacillus and other bacteria, a colony-free “clean zone" had appeared, which once again confirmed the presence of an antimicrobial substance in nutrient media around Bacillus cereus. The influence of Bacillus cereus ATCC 14579 on different bacteria was significantly different. Thus, in our experiments, Bacillus cereus prevented the growth of Staphylococcus epidermidis, Streptococcus pyogenes and Streptococcus agalactiae. Bacillus cereus also showed antagonism to Escherichia coli and Pseudomonas aeruginosa. For other bacteria tested, antagonism was not observed.

Thereby, based on the above facts, it can be assumed that bacteria Bacillus cereus ATCC 14579 was characterized by a wide spectrum of antibacterial activity against staphylococci and streptococci and some Gram-negative bacteria. To confirm this theory, as well as to obtain new antibacterial substances, further studies are expected using microbiological and biotechnological methods.

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