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Partial Characterisation of Bacteriocins Produced by *Bacillus cereus* Isolates from Milk and Milk Products

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Summary

Thirty one (19.2 %) out of 161 *Bacillus cereus* isolates from raw milk and milk products were found to produce proteinaceous substances which inhibit the growth of other *B. cereus* isolates. The detection of antibacterial activity depended on medium and method used. Bactericidal activity was detected in 23 (14 %) or 19 (12 %) of the tested strains on the triptic soya agar and brain-heart infusion with glucose, respectively, while 11 (7 %) of the strains produced bactericidal substances on both media. Nineteen percent of isolates from raw milk and 20 % of isolates from milk products were found to produce bacteriocins. Four *B. cereus* isolates inhibited the growth of individual test strains belonging to *B. licheniformis*, *B. subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus helveticus* and *L. casei* species. The bacteriocins of four *B. cereus* isolates were studied in more detail. The production and activity of these substances were detected in stationary-phase of bacterial culture. Two of them were stable after heating at 60 °C, while only one was stable after heating at 75 °C for 15 minutes. All of them were active over a range of pH=3–10. The apparent molecular weights of four bacteriocins detected by SDS-PAGE electrophoresis were in the range of 1 to 8 kDa.

Key words: bacteriocins, *Bacillus cereus*, milk, milk products, SDS-PAGE electrophoresis

Introduction

Bacillus cereus is one of around 60 representatives of the widely varied *Bacillus* genus. Along with the very similar species *B. mycoides*, *B. thuringiensis* and *B. anthracis*, it comprises the so called »*Bacillus cereus* group«. The differences between these four species are very small. *B. cereus* is found frequently as a saprophyte in soil, water, vegetation and air, from where it is easily transferred to food, either from the original raw material or during the food processing. It is common in dried foodstuffs, spices, cereals, meat, eggs, milk and milk products, cooked and inappropriately kept food *etc*. (*1–3*). The colonisation of different ecological niches is enabled by its extremely good adaptability and resistance to various influences. *B. cereus* produces endospores that survive pasteurisation and are also resistant to various disinfectants. It also forms enzymes such as lipases, proteases, xylanases and others. In milk and milk products, it decomposes casein into peptides and amino acids, and milk fat into free fatty acids, thus degrading the quality of milk products and shortening their shelf life. *B. cereus* produces different types of toxins, hemolysins and phospholipases. Three types of diarrheal enterotoxins have been discovered so far, with the most research done on haemolytic (HBL) and non-haemolytic diarrheal enterotoxin (NHE). The emetic syndrome is a consequence of emetic toxin formation in food (*4–8*). *B. cereus* and some

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closely related species from the genus *Bacillus* have several features including the production of various biologically active metabolites *i.e.* antibiotics, proteinases and bacteriocins that make them attractive candidates for biological control agents. It is well known that most, if not all, bacteria species are capable of producing a heterogeneous array of molecules in the course of their growth *in vitro* (and presumably also in their natural habitats) that may be inhibitory to other bacteria (*9*).

Bacteriocins are proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer microorganisms. They are heterogeneous compounds which vary in molecular weight, biochemical properties, activity spectra and mechanism of action (*10*). These polypeptide antibiotics can possess bactericidal, fungicidal, metal-chelating, and immunomodulating activities. They are frequently found as secondary metabolites produced by various microorganisms, such as the Gram-positive bacteria of the genus *Streptomyces*, lactic acid bacteria and genus *Bacillus* (*10–12*). Bacteriocin production in lactic acid bacteria has been extensively studied. Only limited data exist on bacteriocins from *Bacillus* spp., in particular from *B. cereus*. A plasmid-linked antilisterial bacteriocin, named coagulin, produced by *B. coagulans* I4 has been reported by Marrec *et al.* (*13*). *B. subtilis* JH642 and a wild strain *B. subtilis* 22A produce also an antilisterial peptide that was purified by anion-exchange and gel filtration chromatography (*14*). Thuricin 7 is produced by *B. thuringiensis* (*15*) while *B. licheniformis* 26-103RA strain produces bacteriocin called lichenin (*16*). Naclerio *et al.* (*17*) isolated the bacteriocin cerein-producing strain *B. cereus* GN105.

The aim of our study was to investigate the percentage of *B. cereus* strains isolated from raw milk and milk products which produce bacteriocins. The nature of these substances and their inhibitory activity were investigated as well.

Materials and Methods

Bacterial strains and media

One hundred and sixty one *B. cereus* strains were isolated from raw milk taken directly from cow udders (total 5 strains), from bulk raw milk taken from transportation tanks at the entrance to a dairy (total 92 strains) and from various products from nine Slovenian dairies (total 64 strains). Most frequently, the samples of pasteurized milk, pasteurized cream, milk powder, curd and ice cream were contaminated with *B. cereus*. The strains, which were studied in greater detail, were isolated from bulk raw milk (*B. cereus* 30/11, 8/2 and 6/10), from milk powder (*B. cereus* 15/5) and from pas-

Table 1. Indicator and test strains for determination of bacteriocin activity, their source, media and incubation conditions

Bacterial strain	Sign	Source	Medium	Incubation conditions (°C / h)
Lactobacillus acidophilus	LF221	IM[1]	MRS	37 / 24–48
Lactobacillus helveticus	ATCC 16009	ATCC[2]	MRS	42 / 24–48
Lactobacillus casei	ATCC 393	ATCC[2]	MRS	37 / 24–48
Bacillus licheniformis	BL1	IM[1]	BHI	30 / 18–24
Bacillus subtilis	BS1	IM[1]	BHI	37 / 24–48
Bacillus subtilis	ATCC 663	ATCC[2]	BHI	37 / 24–48
Bacillus stearothermophylus var. *calidolactis*	C 953	Merck (kat.no.11499)	BHI	37 / 24–48
Escherichia coli	ATCC 11229	ATCC[2]	BHI	37 / 24–48
Enterococcus faecalis	EF1	TNO[4]	BHI	37 / 24–48
Pseudomonas aeruginosa	PA	VF[3]	GSP	35 / 72
Staphylococcus aureus	ATCC 25923	ATCC[2]	BHI	37 / 24–48
Staphylococcus aureus	SA	TNO[4]	BHI	37 / 24–48
Yersinia enterocolitica	YE1	IM[1]	NA	25 / 48
Pseudomonas fluorescens/putida	PFP	IM[1]	GSP	25 / 5 days
Aeromonas sp.	AE	IM[1]	GSP	25 / 5 days
Sacharomyces sp.	SC	IM[1]	YDC	25 / 5 days
Strains *B. cereus*		Isolates from milk and milk products, IM[1]	BHIG, TS	30 / 18–24

[1] IM: Institute of Dairying, Zootechnical Dept. University of Ljubljana, Slovenia
[2] ATCC: American Type Culture Collection, Rockville, USA
[3] VF: Veterinary Faculty, University of Ljubljana, Slovenia
[4] TNO: Nutrition and Food Research, Zeist, The Netherlands
Media and incubation purposes according to IDF and ISO standards, Reinheimer (1990), Bridson (1993) and Merck (1994) (*18–25*)
MRS: De Man, Rogosa and Sharpe broth and agar (Merck, Darmstadt, Germany) (*22*)
NA: Nutrient agar
BHI: Brain-heart infussion medium (Biolife)
BHIG: Brain-heart infussion medium with glucose (Biolife) (*18,23*)
GSP: medium according to Kielwein (Merck) (*25*)
YDC: yeast dextrose medium with chloramphenicol (*24*)

teurized milk (*B. cereus* 8/10). Colony morphology, cell-morphological and physiological characteristics were determined using conventional procedures (*18*).

Hydrolysis of lecithin was detected on *B. cereus* selective agar (PEMBA, Biolife, Milano, Italy) supplemented with egg yolk emulsion and polymyxin B (Biolife). Haemolytic activity was determined on blood agar (tryptic soy agar, Biolife supplemented with 5 % defibrinated sheep blood). The plates were incubated at 30 °C for 24–48 h. Milk isolates were identified with the API 50 CHB and API 20 E test systems using the identification programme V.2.0. (BioMerieux, RCS Lyon, France) and BBL Crystal (Becton Dickinson, Cockeysville, MA, USA). All test strains of *B. cereus* were used as indicator strains as well in the cross-sensitivity testing. Indicator strains *B. licheniformis* BL1, *B. subtilis* BS1, *Yersinia enterocolitica* YE 1, *Pseudomonas fluorescens/putida* PFP, *Aeromonas* sp. AE and *Sacharomyces* sp. SC were isolated and identified at the Institute of Dairying, Zootechnical Dept., Biotechnical Faculty, University of Ljubljana, Slovenia. Tested and indicator strains were identified by identification systems API 50 CH, API 20 E, API 10 S, API 20 C (BioMerieux). The bacterial strains, their origin and growth conditions are listed in Table 1. All strains were stored in appropriate media with 20 % glycerol at –80 °C.

Methods

For detection of antimicrobial substances produced by *B. cereus* strains, the deferred agar spot test (DAS) (*9*,*26*) and agar well diffusion assay (AWD) (*10*) were used.

Detection of antimicrobial activity by the deferred agar spot test (DAS)

This method was carried out on tryptic soya agar or broth (TS) (pH=7.3 ± 0.2) (Biolife) and brain heart infusion agar or broth (Biolife) with 0.1 % glucose (BHIG) (pH=7.4 ± 0.2).

Ten μL of culture of *B. cereus* test strains grown for 7 to 8 h in TS and BHIG broth were spot inoculated on the surface of TS and BHIG agar, respectively. After 18–24 h of incubation at 30 °C, a soft overlay of media TS, BHIG, MRS or YDC (5 mL, 0.75 % agar), suitable for indicator strains, inoculated with 100 μL of indicator culture in the stationary phase (approximately 10^5 cells/mL), were poured over the surface of spot inoculated TS or BHIG agar. Inhibition zones were observed after 24–48 h of incubation under appropriate conditions for each indicator strain (Table 1). Clear zones of inhibition with sharp edges around spots were considered as positive results. The measurements were recorded on three or more repetitions.

Detection of antimicrobial activity by agar well diffusion assay (AWD)

The overlay agar inoculated with indicator microorganisms, as described in the DAS assay, was poured onto the BHIG agar plates. Wells of 5 mm diameter were cut into the agar and filled with 50 μL of crude bacteriocin preparation, prepared from overnight culture by centrifugation at 3500 g/15–20 min, neutralised with 5 mol/L NaOH to the final pH=7.0 and then filter-sterilised (*27*,*28*). Plates were pre-incubated at 4 °C for about 2 h to allow diffusion of any inhibitory metabolites into the surrounding agar, and then incubated at the optimum growth temperature of the indicator microorganism. The plates were examined for a clear zone in the agar surrounding the well. The measurements were recorded in three or more repetitions.

Sensitivity of antimicrobial substances to proteolytic and other enzymes

The enzymes and buffers used in this part of the experiment were the following: catalase (Sigma, Chemical Co., St. Louis, MO, USA) 21 000 units/mg dry matter in 0.01 mol/L potassium phosphate buffer, pH=7.0; trypsin (Fluka Chemie, Buchs, Switzerland) 94 units/mg dry matter in 0.1 mol/L Tris HCl, pH=8.0 with 0.01 mol/L $CaCl_2$; protease VIII (Sigma) 13.5 units/mg dry matter in 0.05 mol/L Tris-HCl buffer, pH=8.0; proteinase K (Sigma) 1.7 units/mg dry matter in 0.1 mol/L sodium phosphate buffer, pH=7.0; phospholipase C (Sigma) 10 units/mg dry matter in 0.05 mol/L Tris HCl, pH=7.0 and 0.01 mol/L $CaCl_2$ solution; amyloglucosidase (Sigma) 51 units/mg dry matter in sodium phosphate buffer, pH=7.0; lipase (Sigma) 8.6 units/mg dry matter in 0.05 mol/L Tris HCl, pH=8.0 and 0.01 mol/L $CaCl_2$. Working solutions of enzymes in suitable buffers were 1 mg/mL. All solutions were sterilised by microfiltration, using sterile microfilters, 0.45 μm, Minisart (Sartorius, Göttingen, Germany) (*29*,*30*).

The first procedure included application of the neutralised sterile supernatants of tested strains *B. cereus* into the wells cut into the agar, as described for the AWD assay. A second well was cut into agar near the well filled with the bacteriocin preparation, at a distance where the edge of the inhibition zone was expected, and filled with 10 μL of working solution of enzymes with the concentration of 1 mg/mL of suitable buffer. The buffers without enzymes were used as negative controls. Reduced zones of inhibition on the side with enzyme wells indicated the sensitivity of bacteriocins on the enzymes used. The second procedure included the addition of enzyme solutions at a final concentration of 100 μg/mL to 150 μL of neutralised sterile supernatants of tested *B. cereus* strains. The samples were incubated for about 1 h at 37 °C (proteinase VIII, lipase, phospholipase C), at 42 °C (proteinase K) and at 25 °C (trypsin and catalase) (*17*,*29*). The buffers without enzymes and buffers with enzymes but without supernatants were used as negative controls (*31*). The supernatants without added enzymes were used as positive control. After 1 h of incubation, the samples were heated at 95 °C for 5 min to inactivate the enzymes, then cooled in icewater. Bacteriocin activity was surveyed by the AWD method (*17*). The trials were performed at least three times.

After the results were obtained by DAS and AWD assays, four *B. cereus* strains (*B. cereus* 15/5, *B. cereus* 8/10, *B. cereus* 30/11 and *B. cereus* 8/2) were chosen, which continuously produced larger amounts of bacteriocins on both media (BHIG and TS) for additional tests. For all further investigations *B. cereus* 10/6 was chosen as the indicator strain.

Sensitivity of bacteriocins to heat

The neutralised sterile supernatants of tested strains *B. cereus* 15/5, 8/10, 30/11 and 8/2 as prepared for the AWD assay were heated at 45, 60, 75 and 90 °C for 15 min and then cooled in icewater. Bacteriocin activity was determined with the AWD assay (*17*).

Activity of bacteriocins at different pH values

About 10 mL of supernatants of test strains *B. cereus* 15/5, 8/10, 30/11 and 8/2 prepared as described above for the AWD assay were adjusted to pH=2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with 5 mol/L NaOH and with concentrated HCl. BHIG broth, adjusted to the same pH values, served as a control. After 5 h of incubation at 30 °C, the supernatants were neutralised and the activity of bacteriocins in the supernatants was detected using the AWD assay (*17*,*32*). Measurements were recorded in three or more trials.

Growth kinetics and bacteriocin production

Strains *B. cereus* 15/5, *B. cereus* 8/10, *B. cereus* 30/11 and *B. cereus* 8/2 were inoculated in the BHIG broth and incubated at 30 °C with constant stirring. After 2, 4, 6, 8, 10, 12, 14, 16 and 24 h of incubation, the culture samples were taken and plated on BHIG agar. At the same time, the samples were taken for the determination of bacteriocin activity of supernatants by DAS and AWD assays.

Direct detection of bacteriocins on gel by SDS-PAGE electrophoresis

The concentrated bacteriocin preparations were made from cell-free supernatants of BHIG cultures.

After bacterial cells had been removed from the 24-h culture by centrifugation at 3500 g for 20 min, the supernatants were neutralised, concentrated 15-fold by ultra-filtration with a Minitan™ S unit (Millipore Co., Bedford, MA, USA), using regenerated cellulose sheet (with permeability 5 kDa), and sterilised filter (45 μm-filter Minisart, Sartorius).

The supernatants were concentrated also by dialysis in benzyl-cellulose dialysis sacks (Sigma, MWCO=1.2 kDa). The dialysis occurred against polyethyleneglycol 20000 (Fluka). The concentrated BHIG broth served as a negative control.

SDS-PAGE electrophoresis

The Schägger and von Jagow method (*33*) was used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A 15-μL aliquot of the 15-fold concentrated bacteriocin samples was mixed with 15 μL of 2-fold concentrated sample buffer and boiled for 5 min. Two molecular weight standards were: MW-SDS-17 S (Sigma), 2510–16950 Da and lysozyme (Sigma) with molecular weight 14300 Da. Electrophoresis was performed in a vertical slab gel apparatus (Amersham Biosciences Europe GmbH, Freiburg, Germany). The gel was composed of 12.5 cm layer of 16.5 % acrylamide separating gel, 1.5 cm layer of 10 % acrylamide spacer gel and 1 cm layer of 4 % acrylamide stacking gel. Electrophoresis was carried out for 9 to 10 h at a constant current of 35 mA in the stacking gel, at 50 mA in the spacer gel, and at 60 mA until the end. After electrophoresis, the gel was divided into two parts. The first half, with standard samples and bacteriocin samples, was stained with Coomassie brilliant blue G 250 stain (Sigma) according to Anderson (*34*). The other half of the gel, which was assayed for bacteriocin activity, was fixed with 20 % propanol and 10 % acetic acid, and washed in sterile water as described previously (*35*). The gel was then placed in a sterile petri dish and overlaid with soft BHIG agar, seeded with indicator strain *B. cereus* 10/6 in a concentration of 10^5 CFU/mL (Colony Forming Units per mL). The plate was incubated at 30 °C for 24 h, examined for the location of the growth inhibition zone and compared with the stained part.

Results

Antibacterial activity of B. cereus strains isolated from milk and milk products

The inhibitory activity of each of 161 *B. cereus* strains was detected against at least 15 others in cross-sensitivity testing. Each strain that showed inhibitory activity against at least three indicator strains was considered in our experiment as potential bacteriocin producer. In all cases we included the catalase treatment and neutralisation of the supernatant to exclude the effect of hydrogen peroxide or low pH value on inhibitory activity.

Thirty-one out of 161 (19.2 %) *B. cereus* isolates produced antimicrobial substances on the TS and BHIG media that inhibited the growth of other *B. cereus* isolates. Positive results were found in 23 (14.3 %) and in 14 (8.7 %) cases with the DAS and AWD methods, respectively. In six cases (3.7 %) bactericidal activity was demonstrated by both methods. The differences in the production of bactericidal substances on two different media, TS and BHIG, were observed. On the TS medium, the bactericidal activity was found in 23 tested strains (14.3 %) and on the BHIG medium, in 19 strains (11.8 %). Only eleven strains (6.8 %) produced bactericidal substances in both media. The bactericidal substances of all 31 out of 161 strains tested were inactivated with one or more proteolytic enzymes, indicating their proteinaceous nature.

Antibacterial activity of B. cereus strains against species of microorganisms other than B. cereus

The tested *B. cereus* isolates that were shown to produce antibacterial substances, active against at least three indicator strains from the same species, were tested further against indicator strains belonging to other species.

Four out of 23 strains, which showed antibacterial activity against the strains *B. cereus* with DAS method, inhibited also the growth of indicator strains belonging to other bacterial species (Table 1). From four *B. cereus* isolates, which were studied in our work in greater detail, only *B. cereus* 30/11 produced the antibacterial substances against other bacterial species tested (Table 2).

Table 2. Antibacterial activity of *B. cereus* strains against species of microorganisms other than *B. cereus*

Indicator strains	*B. cereus* test strains						
	30/11	15/5	8/10	8/2	4/9	4/10	10/11
Lactobacillus acidophilus LF221	–	–	–	–	–	–	–
Lactobacillus helveticus ATCC 16009	+	–	–	–	–	–	–
Lactobacillus casei ATCC 393	+	–	–	–	–	–	–
Bacillus licheniformis BL1	–	–	–	–	+	+	+
Bacillus subtilis BS1	–	–	–	–	+	+	+
Bacillus subtilis ATCC 663	+	–	–	–	+	+	+
Bacillus stearotherm. var. *calid.* C953	–	–	–	–	–	+	+
Escherichia coli ATCC 11229	–	–	–	–	+	+	–
Enterococcus faecalis EF1	–	–	–	–	+	+	–
Pseudomonas aeruginosa PA	–	–	–	–	–	–	–
Staphylococcus aureus ATCC 25923	–	–	–	–	+	+	–
Staphylococcus aureus SA	–	–	–	–	–	–	+
Yersinia enterocolitica YE1	–	–	–	–	–	–	+
Pseudomonas fluorescens/putida PFP	–	–	–	–	–	–	–
Aeromonas sp. *AE*	–	–	–	–	–	–	–
Sacharomyces sp. SC	–	–	–	–	–	–	–

+ antibacterial activity; – no antibacterial activity

Sensitivity of bacteriocins produced by B. cereus 15/5, 30/11, 8/10 and 8/2 strains on enzymes

In the second part of our experiment, four *B. cereus* strains, 30/11, 15/5, 8/10 and 8/2, which constantly produced bacteriocins on the BHIG and TS media, were studied in more detail. Treatment with trypsin caused a reduction of antibacterial activity of substances produced by *B. cereus* 8/2. Treatment with proteinase K and protease caused a reduction of antibacterial activity of substances produced by all of the four *B. cereus* strains tested; lipase inactivated the antibacterial substances produced by strain 15/5; while amyloglucosidase inactivated the antibacterial substances produced by strains 8/10 and 8/2. Buffers and enzyme solutions alone had no effect on the indicator strain *B. cereus* 10/6. The treatment with catalase had no effect on the bacteriocin activity tested, indicating that hydrogen peroxide was not involved in antimicrobial activity (Table 3). Based on the demonstrated sensitivity of antimicrobial substances to at least one proteolytic enzyme, they were still considered as bacteriocins.

Table 3. Sensitivity to enzymes of antimicrobial substances in the supernatant of *B. cereus* 30/11, 15/5, 8/10 and 8/2 cultures

Enzymes	*B. cereus* strains			
	30/11	15/5	8/10	8/2
Catalase	–	–	–	–
Trypsin	–	–	–	+
Protease	+	+	+	+
Proteinase K	+	+	+	+
Phospholipase C	–	–	–	–
Amyloglucosidase	–	–	+	+
Lipase	–	+	–	–

+ bacteriocin inactivation by treatment; – bacteriocin resistant to treatment

Effect of pH values and heating on the activity of antimicrobials in culture supernatants

The antimicrobials in the supernatants of all four tested strains showed stability at pH values ranging from pH=3.0–10.0. Total loss of antimicrobial activity of bacteriocins produced by strains 30/11 and 8/2 was detected only after 5 h of incubation at 30 °C in broth adjusted to pH=2.0 (Table 4).

After heating for 15 min at 45 °C the activity of the tested bacteriocins was the same as that of the untreated control samples. Heating for 15 min at 60 °C partially reduced the activity of bacteriocins produced by strains 15/5 and 8/2. Only bacteriocins produced by the *B. cereus* 30/11 strain were not affected by heating for 15 min at 75 °C. Heating at 90 °C for 15 min affected the activity of all four tested bacteriocins (Fig. 1).

Growth kinetics and bacteriocin production

Bacteriocin production and secretion was observed when test strains *B. cereus* 15/5, 30/11, 8/10 and 8/2 were grown in glucose-supplemented BHI broth at 30 °C. As shown in Figs. 2a, 2b, 2c and 2d for four tested bacteriocin producers, bacteriocin activity was not detected earlier than at the beginning of the stationary phase. In the case of 30/11 strain, bacteriocin detection limit was reached after 10 h, while for the other three strains, bacteriocins were detected during the next sampling (6 h later). A decrease of bactericidal activity after 24 hours occurred only in strain 30/11 culture and it was accompanied with the decrease of the viable cells number.

SDS-PAGE electrophoresis

In the supernatants of *B. cereus* 8/10 and 8/2 cultures, the concentration of bacteriocins was obtained using ultrafiltration membranes of MWCO 5 kDa, while in the *B. cereus* 30/11 and 15/5 strains dialysis against PES

Table 4. Effect of pH values on bacteriocin activity

pH value	Inhibition zone of *B. cereus* strains /mm*			
	30/11	15/5	8/10	8/2
2.0	0	2	2	0
3.0	5	7	5	6
4.0	6	7	5	6
5.0	6	7	5	6
6.0	6	7	5	6
7.0	6	7	5	6
8.0	6	7	5	6
9.0	6	7	5	6
10.0	6	7	5	6

*Activity was expressed as diameter (mm) of inhibition zones using the AWD method (BHIG medium); the results are mean values of triplicate tests

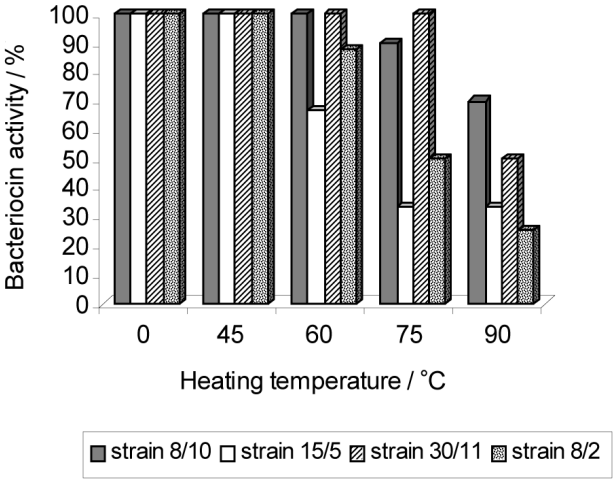

Fig. 1. Effect of heating on the activity of bacteriocins produced by *B. cereus* strains 15/5, 30/11, 8/10 and 8/2. The bacteriocin activity was determined by AWD assay and expressed as the percent of retained activity in comparison with the activity of untreated samples. The mm of inhibition zones of untreated samples represented 100 % of activity

(MWCO of dialysis bags 1.2 kDa) resulted in better yield. The preparations of 2–2.5 times concentrated bacteriocin were used for determination of molecular weight by SDS-PAGE electrophoresis. After electrophoresis the polyacrylamide gel containing bacteriocin samples was cut into two vertical parts. The part containing the samples and molecular weight markers was stained, while the remaining part, which contained only the samples, was fixed and used for direct detection of antimicrobial activity. As shown in Fig. 3, the bactericidal activities of the bacteriocins of *B. cereus* 30/11, 15/5, 8/2 and 8/10 were detected in the parts of gel corresponding to the molecular weights of about 2.5–3.5 kDa, 1–2.5 kDa, 4–6 kDa and 5.5–8 kDa, respectively (Fig. 3).

Discussion

A lot of studies on bacteriocins produced by lactic acid bacteria have been reported recently. However, there is not so much data available about bacteriocins produced by *Bacillus* spp. bacterial strains, although bacteriocin-like inhibitors produced by *B. stearothermophilus*, *B. licheniformis*, *B. thuringiensis*, *B. subtilis* and *B. megaterium* are well known (*9*,*14*,*15*,*36*).

In our study bacteriocin-like substances produced by *B. cereus* strains, isolated from milk and milk products were tested. About 31 (19.2 %) out of 161 *B. cereus* isolates produced substances that inhibited the growth of other *B. cereus* isolates. The results of bacteriocin production and activity varied depending on the method (DAS or AWD) and media (TS or BHIG) used.

It is well known that bacteriocins produced by lactic acid bacteria are very often sensitive to trypsin, while the sensitivity to other enzymes varies (*11*). *B. cereus* bacteriocins presented in this study were not typical in this respect, as three of them were not sensitive to trypsin in tested concentrations but were inactivated by protease and proteinase K. The inactivation of bacteriocin activity by amyloglucosidase in two strains and by lipase in one strain might be an indication that beside proteinaceous subunit, some lipid or carbohydrate components are involved in the antibacterial activity as well. However, although a group of complex bacteriocins was proposed by Klaenhammer classification (*10*), no such bacteriocins were isolated so far. Although bacteriocins can be present in complexes with other macromolecules in the crude extracts due to cationic and hydrophylic interactions, these complexes are usually disintegrated during purification, while the bacteriocin activity is maintained (*37*). Nevertheless, it is possible that more than one bacteriocin substance produced by the same tested strains is present in the crude extracts. For additional information about their structure, more detailed examinations have to be made, particularly in view of the fact that some authors have expressed that antibacterial substances produced by *B. cereus* strains might be identical to phospholipase A (*9*).

Some of the test strains produced bacteriocins which inactivated the growth of strains from other genera (Table 1), including some lactic acid bacteria and pathogens, while the bacteriocin called cerein was shown to be active exclusively against other *B. cereus* strains and inactive against all other bacterial species tested (*17*).

About 19 % of isolates from raw milk and 20 % of isolates from milk products produced bacteriocins, respectively (data not shown), so it can be supposed that bacteriocinogenic *B. cereus* strains are equally represented in raw milk and milk products.

Out of the 31 bacteriocin-producing strains, four were chosen for a more detailed examination. These bacteriocins were produced when the strains were grown in BHIG and TS media. All four tested bacteriocins produced by *B. cereus* strains 30/11, 15/5, 8/10 and 8/2 showed stability at a wide range of pH values from pH=3.0–10.0 at 30 °C. This is different from nisin, which is unstable at neutral and alkaline pH values (*38*).

In comparison with some of the bacteriocins produced by lactic acid bacteria, *B. cereus* bacteriocins were not extremely heat stable (Fig.1). For example, bacteriocins produced by *L. acidophilus* LF221 or lacticin NK24 from *Lactococcus lactis* could at least partially preserve

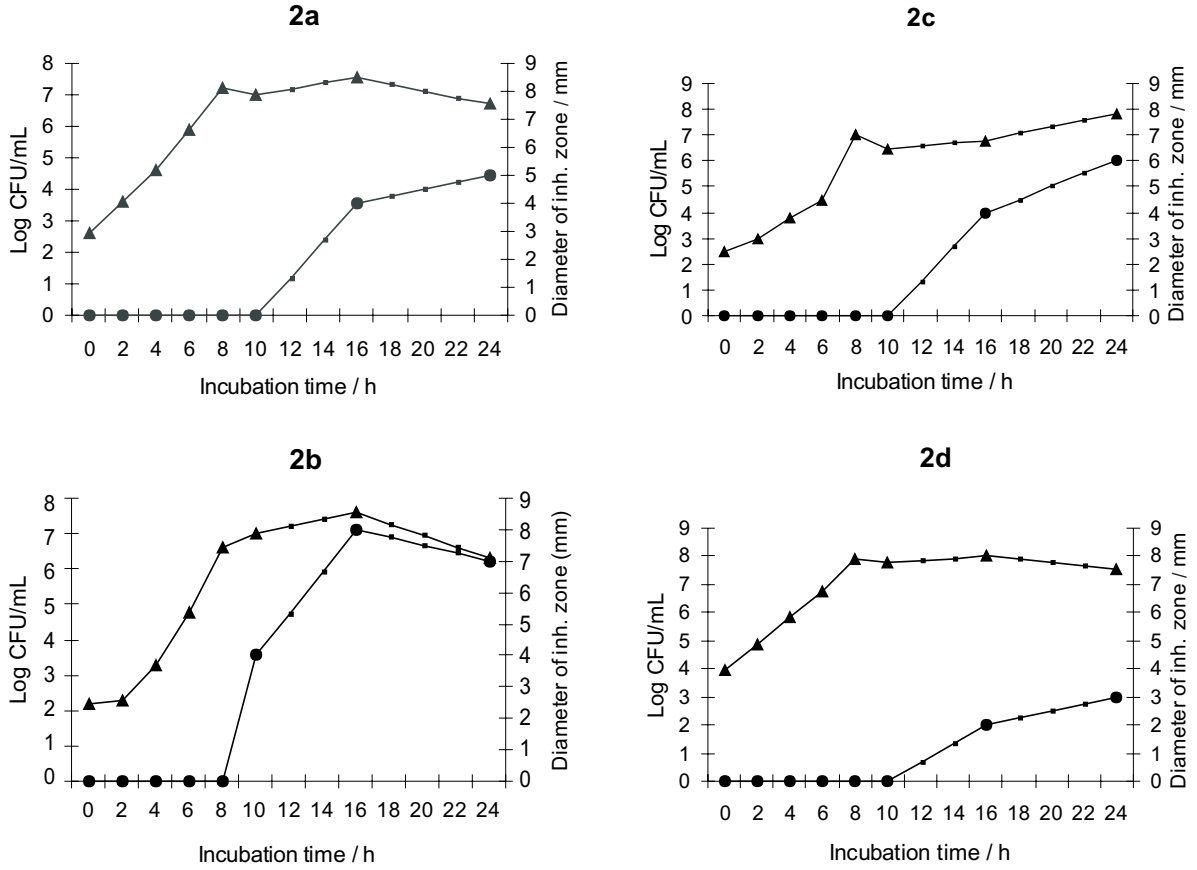

Fig. 2a-d. Bacteriocin production in the course of the bacterial population growth in the strains *B. cereus* 8/2 (Fig. 2a), 30/11 (Fig. 2b), 8/10 (Fig. 2c) and 15/5 (Fig. 2d). Bactericidal activity was expressed as a diameter of the inhibition zone, determined by AWD method. The viable cell number was expressed as logarithmic values of CFU

their activity, even after heating at 100 °C for 30 min (*32*,*39*). On the other hand, our four bacteriocins had very similar activity characteristics at different pH values with the bacteriocin cerein isolated from *B. cereus* GN105 (*17*), in spite of the differences in heat stability (they were more resistant to heating) and the obvious differences in sensitivity to proteolytic enzymes.

In all four cases bacteriocin production and secretion was observed in the stationary phase, after 10 to 16 h of bacterial population growth in BHI broth at 30 °C. The production of bacteriocins took place successfully in both broth media and on the solid media. Naclerio *et al.* (*17*) also reported the production and activity of bacteriocin cerein by *B. cereus* strain in the stationary phase, while Cherif *et al.* (*15*) reported that thuricin 7, produced by the very closely related species *B. thuringiensis* BMG1.7, was expressed in the exponential growth phase. As it is well known, bacteriocins of lactic acid bacteria, particularly lantibiotics, are usually produced in the exponential phase (*40*).

The molecular weights of bacteriocins produced by *B. cereus* 8/2 and *B. cereus* 8/10 were approximately 4–6 kDa and 5.5–8 kDa, respectively, while the molecular weights of bacteriocins produced by *B. cereus* 15/5 and *B. cereus* 30/11 were lower (approximately between 1–3 kDa and 2.5–3.5 kDa, respectively). Some other bacteriocins, produced by strains from the *Bacillus* genus, like polyfermenticin (*39*), cerein (*17*), and thuricin 7 (*15*),

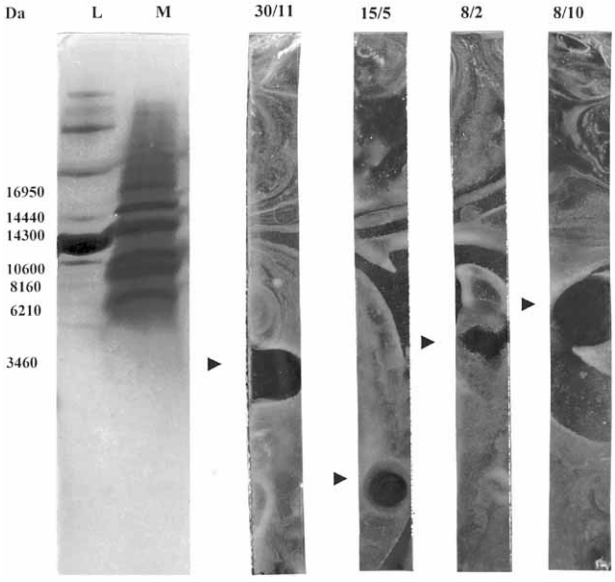

Fig. 3. SDS-PAGE electrophoresis gel of concentrated bacteriocins produced by strains *B. cereus* 30/11, 15/5, 8/2 and 8/10. On the left gel are standards Lysozyme (L) (14.3 kDa) and molecular marker (M) (MW-SDS-17S, Sigma). The other four gels repeat the parts of the SDS-PAGE gels with bacteriocins which were overlayed with indicator strain *B. cereus* 6/10 seeded in the soft BHI medium

have molecular weights ranging between 9 and 14 kDa, which are higher than those of our bacteriocins. Lichenin, produced by *B. licheniformis* 26L-10/3RA (*16*), ericin S (3.442 kDa) and ericin A (2.986 kDa) from *B. subtilis* (*36*) all have similar molecular weights.

Although *B. cereus* is considered as potential pathogen microorganism, the properly identified and tested nontoxic strains are most frequently used as animal probiotics (*41–43*). Another possibility is the purification of bacteriocins, which could be added to food products as a preservative or transfer of genetic material that codifies bacteriocin to a type of food grade microorganism.

Conclusions

On the basis of the presented data, it can be assumed that our tested *B. cereus* strains can produce bacteriocins. To characterise them, more detailed research on their synthesis system, the secretion and regulatory process-including their purification, amino-acid sequencing, *etc*. will have to be done in the future.

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Djelomična karakterizacija bakteriocina proizvedenih u izolatima *Bacillus cereus* dobivenih iz mlijeka i mliječnih proizvoda

Sažetak

Od 161 izolata *Bacillus cereus*, dobivenih iz mlijeka i mliječnih proizvoda, u 31 izolatu (19,2 %) nađene su proteinske supstancije koje inhibiraju rast drugih izolata *B. cereus*. Utvrđivanje antibakterijske aktivnosti ovisi o upotrijebljenom mediju i postupku. Baktericidna aktivnost otkrivena je u 23 (14 %) ispitivanih sojeva uzgajanih na podlozi tripsin-soja-agar ili u 19 (12 %) na podlozi mozak-srce uz dodatak glukoze. Jedanaest sojeva (7 %) proizvelo je baktericidne spojeve na obje podloge. Bakteriocini su utvrđeni u 19 % izolata iz sirovog mlijeka, te 20 % iz mliječnih proizvoda. Četiri izolata *B. cereus* inhibirala su rast pojedinih test sojeva, i to *B. licheniformis*, *B. subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus helveticus* i *L. casei*. Iscrpnije su ispitani bakteriocini iz četiri izolata *B. cereus*. Proizvodnja i aktivnost tih spojeva otkriveni su u stacionarnoj fazi bakterijskog uzgoja. Dvije su aktivne supstancije bile stabilne pri zagrijavanju na 60 °C a jedna je od njih ostala stabilna i nakon zagrijavanja na 75 °C tijekom 15 min. Svi su spojevi bili aktivni pri pH=3–10. Prividna je molekularna masa ovih četiriju bakteriocina, utvrđena SDS-PAGE elektoforezom, iznosila je od 1 do 8 kDa.