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Physical chemical and biological characterization of a new bacteriocin produced by *Bacillus cereus* NS02

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ABSTRACT

Objective: To screen the bacteriocinogenic isolate from buffalo milk and to characterize it on physical, chemical and biological aspects for the application in biopreservation. **Methods:** *Bacillus cereus* (*B. cereus*) was isolated and assessed for its baceteriocinogenic activity. Bacteriocin was produced and purified by ammonium sulphate precipitation, dialysis and gel filtration chromatography. Purified bacteriocin was used to check its antimicrobial activity against food borne bacteria. Effect and stability of bacteriocin was determined with the respect to temperature, pH, enzymes, organic solvents and chemicals. Bacteriocin was also subjected to SDS PAGE analysis to determine its molecular weight. In addition, functional groups exist in the bacteriocin was determined by FTIR analysis. **Results:** *B. cereus* was identified by 16S rRNA sequence analysis. Bacteriocin showed increased activity against all the bacteria used and its activity unit was found to be 51, 200 AU/mL. It was stable to high temperature (100 ℃) and wide range of pH (3–10), sensitive to proteolytic enzymes and resistant to nonproteolytic enzymes. It was low molecular weight (3.5 – 6 KDa) protein and FTIR study revealed the presence of amide group and NH stretching. **Conclusions:** Bacteriocin produced in this study possesses the highest antimicrobial activity against both gram positive and gram negative bacteria thereby it has immense application as biopreservative agent. FTIR proved its peptide nature.

1. Introduction

Control of both pathogenic and spoilage microbes in a variety of foods are important for food quality and safety. Recently, usage of food additives has been decreased due to the safety concerns and biopreservation has become a topic of interest. It is an alternative technique to chemical additives for increasing life time and safety of foods by using natural microflora and their antimicrobial products[1, 2] and they are recognized as safe status (GRAS)[3]. Antagonistic effect produced by LAB towards other organisms may also play an important role in maintaining a proper microbial balance in intestinal tract and preserving certain foods and this effect is due to the production of antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocin or related substances[4].

Bacteriocins are ribosomally synthesised, low molecular weight peptides that are heat stable and sensitive to proteolytic enzymes[5,6]. Bacteriocin generally exhibit their antimicrobial action by interfering with the cell wall or membrane of target organisms, either by inhibiting cell wall synthesis or causing pore formation, resulting in death[7]. There is a growing consumer demand for processed food products containing lower levels or no chemical additives, leading to indigenous research studies in the field of screening bacteriocin as food preservatives. In recent years, there have been many reports on bacteriocins produced by LAB. However, nisin and pediocin PA–1 are commercially available and used in the food industries as biopreservative due to they are received GRAS status[8]. A potentially novel pediocin NV5 was found active against some species of *Enterococcus*, *Leconostoc*, *Staphylococcus*, many of which are associated with food spoilage and food related health hazards[9].

The new bacterioicin producing *Lactobacillus fermentum*

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(*L. fermentum*) KN02[10] and *L. fermentum* UN01[11] has been proved its biopreservative effects on milk, mushroom, fruit juice and fish. Antimicrobial agents are also produced by other Gram positive bacteria including *Bacillus* sp: *Bacillus subtilis (B. subtilis)*[12], *Bacillus thruringiensis (B. thruringiensis)*[13], *Bacillus licheniformis (B. licheniformis)*[14], *Bacillus thermoleovorans (B. thermoleovorans)*[15] and *Bacillus cereus* (*B. cereus*)[16] *Bacillus coagulans (B. coagulans)*[17] and *Bacillus amyloliquifaciens (B. amyloliquifaciens)*[18]. In spite of considerable studies taken by scientists to collect data on *Bacillus* bacteriocins, the importance and the industrial value of those bacteriocins has been largely underestimated and has attracted little attention compared to those produced by LAB. So this study primarily aimed at screening for bacteriocinogenic Bacillus strain from buffalo milk to reveal and evaluate the characterisation of their bacteriocin.

2. Materials and methods

2.1. Bacterial strains

Buffalo milk sample was collected aseptically and 0.1 mL portion of respective dilution of homogenates in 0.5% peptone water was spread on the MRS agar (De Man – Rogosa Sharpe, Hi media, India). Subsequently all the plates were incubated at 37 ℃ for 48 h.

2.2. Screening of bacteriocinogenic strains

Based on the colony morphology, pure culture of each isolate was made. 24 h old culture of each isolate was inoculated into 25 mL of MRS broth and incubated for 24 h. Then, the broth culture was centrifuged at 8 000 rpm for 10 min to collect the cell free supernatant (CFS)[19]. It was used to check the antimicrobial activity against *B. coagulans* (MTCC 1272) as indicator strain.

2.3. Identification of bacteriocinogenic strain

The strain showed better activity than other isolates was subjected to further identification on the basis of 16S rRNA sequence analysis and this was performed at Microbial Type Culture Collection Centre (MTCC), Chandigarh, India.

2.4. Production and purification of bacteriocin

The CEP was first subjected to ammonium sulfate precipitation. Ammonium sulfate was added to CEP until the concentration of 60% (w/v) saturation then the precipitate was settled overnight. This was centrifuged at 6 500 rpm in a refrigerated centrifuge for 30 min to collect the precipitate. It was then dissolved in 10 mL of 0.1 M phosphate buffer (pH 6.0) and dialyzed against 500 mL of 0.1 M phosphate buffer (pH 6.0) at 4 ℃ for overnight[20]. The crude protein was purified by gel filtration chromatography using a cationic resin Sephadex G 100 (Hi media, India). It was loaded onto a column (1.0 cm × 15.0 cm) packed with Sephadex G 100 equilibrated with 0.1 M phosphate buffer (pH 6.0). After washing with buffer (2 times), the elution was performed with a negative linear gradient of 0 to 1.0 M NaCl[21]. The eluted compound was freeze dried using lyophilizer (Merk, India) for further study

2.5. Determination of antagonistic effect of bacteriocin

Purified bacteriocin was obtained by above mentioned method and different volume of the sample (50, 100 and 150 μL) was used to check antimicrobial activity against food borne pathogens *viz*. *Listeria monocytogenes* MTCC 657 (*L. monocytogenes*), *B. coagulans* MTCC 1272, *Staphylococcus aureus* MTCC 737 (*S. aureus*), *Shigella flexeneri* MTCC 1457 (*S. flexeneri*), *Escherichia coli* MTCC 1687 (*E. coli*), *Proteus mirabilis* MTCC 425 (*P. mirabilis*), *Salmonella typhi* MTCC 531 (*S. typhi*), *Klebsiella pneumoniae* MTCC 530 (*K. pneumoniae*) and *Pseudomonas aeruginosa* MTCC 1688 (*P. aeruginosa*) by well diffusion method. All these strains were collected from MTCC and Gene Bank of Institute of Microbial Technology, Chandigarh, India. The antagonistic activity of the bacteriocin was evaluated by measuring the resulting diameters of zone of inhibition in millimeters.

2.6. Bioassay of bacteriocin

The titre was defined as the reciprocal of the highest dilution (2^n) that resulted in inhibition of the indicator lawn. Thus the arbitrary unit (AU) of antimicrobial activity mL^{-1} was defined as $2^n \times 1\,000$ μL/10 μL[22]. To examine the activity units, purified bacteriocin was serially diluted two fold with 0.1 M acetic acid. About 50 μL from each dilution was added into the plates containing a lawn of *B. coagulans* and *S. typhi*.

2.7. Optimum reaction condition

2.7.1. Effect of pH and temperature on purified bacteriocin activity and stability

The effect of pH on the purified bacteriocin was determined by measuring the bacteriocin activity at 37 ℃ at various pH levels in the following buffers: 0.1 M acetate buffer (3–5), 0.1 M phosphate buffer (6–7) and Tris HCl (8–10). The stability of the bacteriocin at various pH was studied by incubating the bacteriocin in various buffers with pH ranging from 3–10 for 24 h at 37 ℃. Then the antimicrobial activity was assayed by well diffusion method. The optimum temperature of the bacteriocin was determined by measuring the bacteriocin activity at various temperatures (40–100 ℃) in 0.1 M phosphate buffer (pH 6.0). The thermal stability was known by incubating the bacteriocin at each specific temperature for 2 h and measuring the antimicrobial activity as above

mentioned method.

2.7.2. Effect of enzymes and organic solvents on bacteriocin activity

Proteolytic enzymes such as papain, pepsin, trypsin, and non proteolytic enzymes such as lipase and amylase were dissolved in phosphate buffer (pH 6) to get a final concentration of 1 and 2 mg/mL. Purified bacteriocin was mixed with each enzyme solution and then incubated at 37 ℃ for 2 h followed by heated in boiling water bath to inactivate the enzymes and tested for antimicrobial activity. Likewise, purified bacteriocin was dissolved individually in the solvents, like acetone, chloroform, ethyl alcohol and methanol (10 mg/mL) and incubated at 37 ℃ for 1 h, and then the solvents were removed by evaporation. The powder form was resusupended in phosphate buffer (pH 6) and tested for antimicrobial activity.

2.8. Synergistic effect of bacteriocin with other chemicals

To determine the synergistic effect of bacteriocin with chelator like EDTA and other chemicals like sodium lactate (NaL) and potassium sorbate (KS), it was separately treated with EDTA (10 mM), NaL (2%) and KS (0.02%)[23]. After 2 h the antimicrobial activity was monitored against gram positives such as *S. aureus*, *L. monocytogenes* and gram negatives such as *E. coli*, *P. mirabilis* and *S. typhi*.

2.9. Mode of action of bacteriocin

To determine whether *B. cereus* bacteriocin showed bactericidal or bacteriostatic action, early log phase culture of *L. monocytogenes* was treated with various concentrations of purified bacteriocin (25 600-200 AU/mL). The viable cells were counted and the OD of the broth culture was taken at 650 nm using a spectrophotometer at every 1h intervals. The viable cells of *L. monocytogenes* were counted by the spread plate method using nutrient agar.

2.10. Protein concentration determination

Protein concentration of purified bacteriocin was determined by Venema *et al*'s[24] method using bovine serum albumin as standard[25].

2.11. FT- IR analysis

A Perkin-Elmer infrared spectrophotometer was used for the investigation of the surface functional groups. The samples with KBr (spectroscopic grade) pellets were prepared in the size of about 10-13 mm in diameter and 1 mm in thickness. The samples were scanned in the spectral range of 4 000-400 cm^{-1}.

2.12. Molecular weight determination

The molecular weight of the purified bacteriocin was determined by SDS-PAGE using the method of Aktypis *et al*[26]. The protein marker (GeNei, India) ranging from 3.0 to 43.0 KDa was used as a standard marker and the protein bands were stained by Coomasiee Brilliant Blue Staining.

3. Results

3.1. Screening and identification of bacteriocinogenic strains

A total of 5 bacteriocinogenic strains were screened from buffalo milk using *B. coagulans* as indicator. Of all the isolates, strain 3 was found to be the best one which showed maximum inhibitory activity. The strain 3 was identified as *B. cereus* by 16S rRNA sequence analysis (NCBI Accession No. JQ904303).

3.2. Production and purification of bacteriocin

24 h old culture of *B. cereus* was used for production of bacteriocin. Crude bacteriocin was produced by salting out and dialysis and it was subjected to gel filtration chromatography for further purification.

3.3. Antagonistic effect and bioassay of bacteriocin

Purified bacteriocin was used for demonstrating their antimicrobial activity against MTCC strains and the result showed that it has increased activity against all the bacteria including gram negatives used. It also proved that higher volume (150 μL) showed better antimicrobial activity than lower volume (50 and 100 μL) (Figure 1). A quantitative study was undertaken to evaluate the activity units of the bacteriocin. It was observed that 51, 200 AU/mL for both the indicators. So there is no difference in activity unit of bacteriocin among the indicators used.

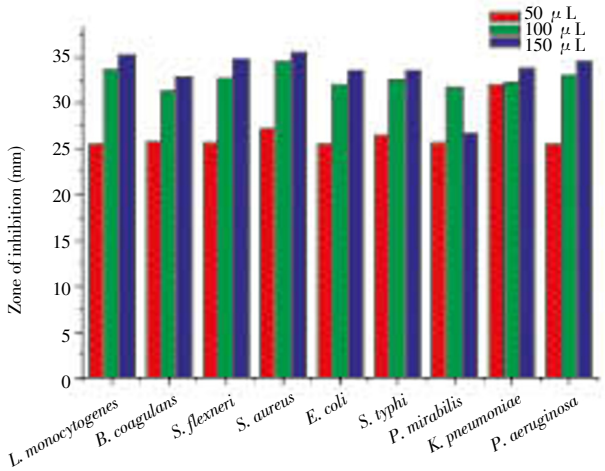

Figure 1. Antimicrobial activity of bacrteriocin against MTCC strains.

3.4. Optimum reaction condition

3.4.1. pH and temperature optimum and stability

B. cereus could tolerate wide range of pH (3–10) with maximal antimicrobial activity at pH 6 (33 and 31.2 mm for *B. coagulans* and *S. typhi* respectively), eventhough the considerable activity was observed from pH 3 to 10 (Figure 2). The stability of bacteriocin remained relatively stable within the pH 6 and 7, with lower values of stability (14.3 and 13 mm for *B. coagulans* and *S. typhi* respectively) at pH 3. Likewise, the bacteriocin was almost resistant to broad range of temperatures (31.3 mm and 12.6 mm for *B. coagulans* at 40 and 100 ℃ respectively) and pertaining maximum activity at 40 ℃ (31.3 mm and 32.0 mm for *B. coagulans* and *S. typhi* respectively) (Figure 3). The purified bacteriocin was stable up to 100 ℃ for 2 h with considerable antimicrobial activity (11.7 mm and 10.2 mm for *B. coagulans* and *S. typhi* respectively).

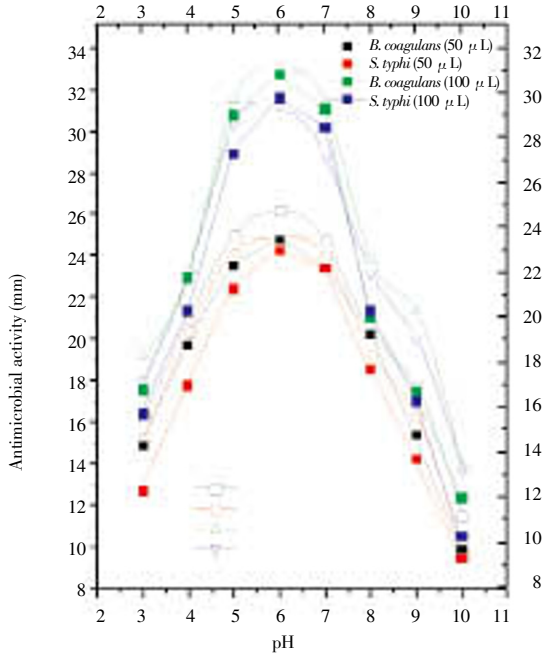

Figure 2. Relative bacteriocin activity and stability at different pH.

3.4.2. Effect of enzymes, organic solvents and other chemicals

The bacteriocin was inactivated by pepsin, proteinase K and trypsin, thereby no inhibition zone around the colonies. These results conclude that the bacteriocin is a proteinaceous compound. The nonproteolytic enzymes like amylase, lipase, diastase and the organic solvents used did not affect the antimicrobial activity of bacteriocin (Table 1).

Analysis of the synergistic effect of bacteriocin with various chemicals showed that the bacteriocin–EDTA combination was found to be a major factor affecting the bacterial growth. The sensitivity pattern of the bacteria order was *S. aureus* > *L. monocytogenes* > *E. coli* > *P. aeruginosa* > *S. typhi*. The results also proved that the chemicals EDTA, NaL and KS showed poor or no antimicrobial activity when tested with above mentioned bacteria (Figure 4).

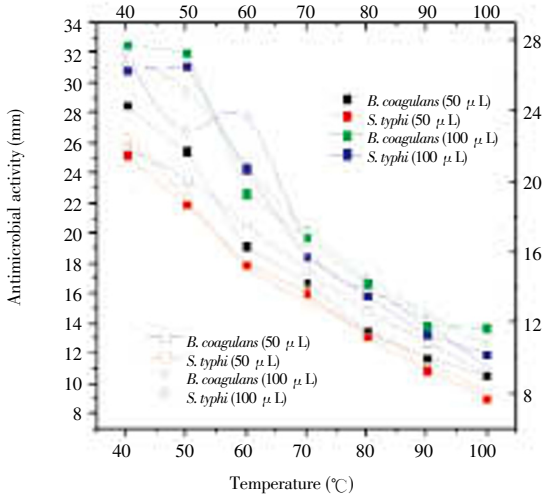

Figure 3. Relative bacteriocin activity and stability at different temperature.

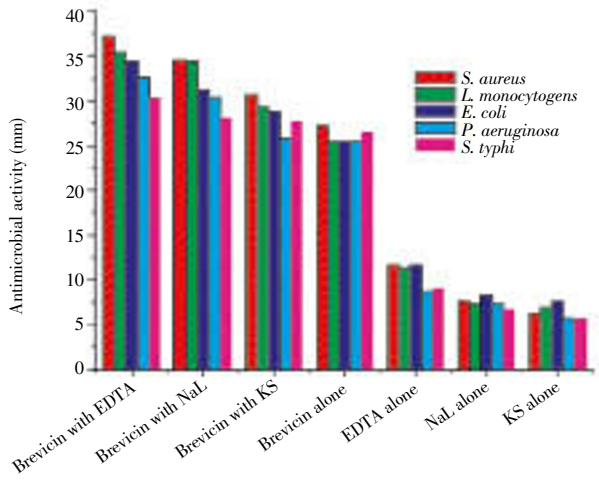

Figure 4. Synergistic effects of bacteriocin with chemicals.

3.5. Mode of action

When the exponential growth phase culture of *L. monocytogenes* was exposed to 25 600AU/mL of purified bacteriocin, the viable cell count was decreased from 9 log to 6.2 log CFU/mL within 1h after the treatment. When the concentration of bacteriocin was increased to 200AU/mL^{-1} the cell count was decreased from 9 log to 3.2 log CFU/mL (Figure 5). The OD of the bacteriocin treated *L. monocytogenes* cell suspensions remained stable after

Table 1
Effect of enzymes and organic solvents in bacteriocin activity.

MTCC strains	Zone of Inhibition (mm)															
	Amylase		Protease		Papain		Trypsin		Diastase		Lipase		Chloroform		Ethanol	
	50 μL	100 μL	50 μL	100 μL	50 μL	100 μL	50 μL	100 μL	50 μL	100 μL	50 μL	100 μL	50 μL	100 μL	50 μL	100 μL
B. coagulans	25.3	30.3	–	–	–	–	–	–	24.3	29.6	22.8	29.3	24.3	30.3	24.3	31.3
S. typhi	22.5	28.7	–	–	–	–	–	–	22.8	27.3	25.3	29.0	22.8	30.7	24.3	30.5

bacteriocin treatment (Figure 6).

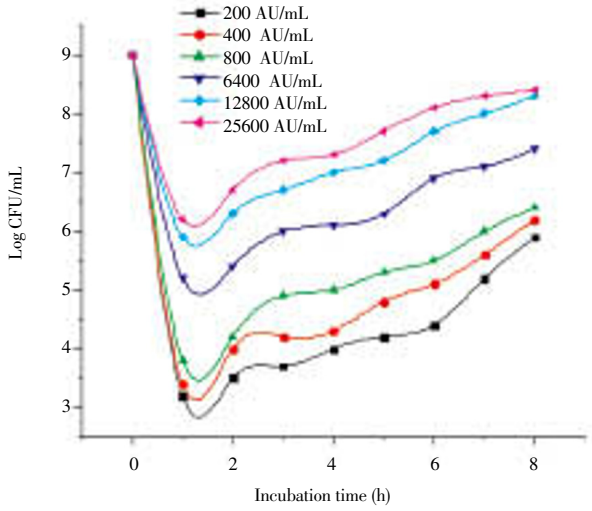

Figure 5. Cell count of *L. monocytogenes* after treating with bacteriocin.

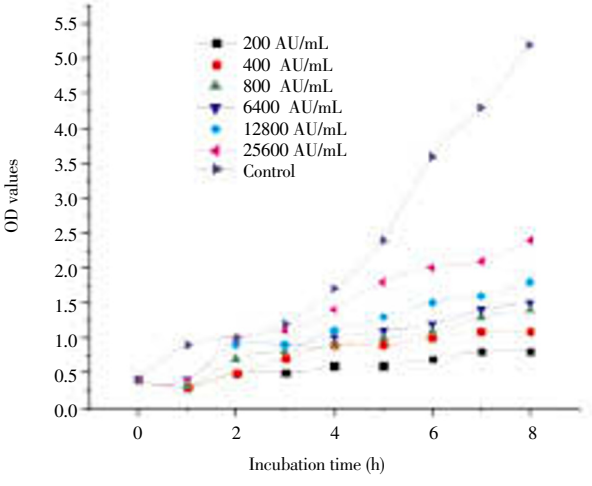

Figure 6. OD values of *L. monocytogenes* broth culture after treating with bacteriocin.

3.6. Determination of protein concentration and molecular weight

The protein concentration of the purified bacteriocin was 2.1 mg/mL. Its molecular weight was found to be within the range of 3.5–6.0 KDa, it indicates that the bacteriocin was low molecular weight protein (Figure 7).

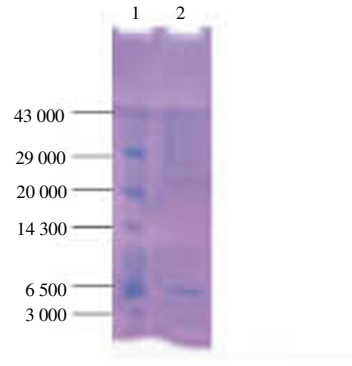

Figure 7. SDS PAGE analysis of bacteriocin.
Lane 1. Protein Marker (Da);
Lane 2. Bacteriocin.

3.7. FTIR analysis

The FTIR spectrum of brevicin observed at 2 858.53 and 2 910.64 cm^{-1} revealed the presence of aliphatic C–H stretching of fatty acids at 1 460.35 and 1 638.90 cm^{-1} bared the presence of amide Ⅰ and amide Ⅱ respectively; at 3 203.79 cm^{-1} showed the presence of aromatic hydrocarbon; at 3 468.35 cm^{-1} revealed the presence of primary and secondary amine (hydroxyl functionality); at 3 569.63 exposed the presence of free hydroxyl group (Figure 8).

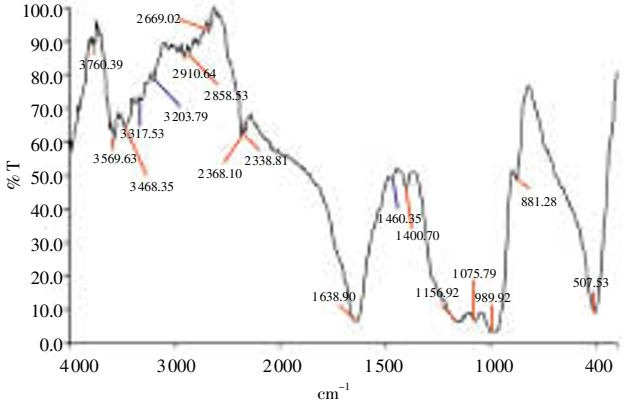

Figure 8. FTIR analysis of bacteriocin.

4. Discussion

Bacteriocins are cationic hydrophobic antimicrobial compounds that are produced by many different species,

including LAB[7]. Bacteriocins produced by LAB are of special interest due to their significant application in food preservation. These compounds mainly prevent the closely related bacteria, although many bacteriocins produced by LAB are also active against unrelated food borne pathogens[27-29]. The present study is aimed at screening the broad spectrum of activity of bacteriocinogenic bacteria from buffalo milk. Their antimicrobial activity was checked and optimized. The bacteriocin produced by *B. cereus* showed better antimicrobial activity against gram positive and negative bacteria. The higher concentration of bacteriocin exhibit increased antimicrobial activity on gram negatives. Because the outermembrane of gram negative bacteria do not allow molecules like bacteriocin to reach its site of action, which is the cell membrane[30]. Used the genera of *Bacillus, Micrococcus, Listeria, Lactobacillus, Enterococcus, Leconostoc, E. coli, Pseudomonas, Salmonella, Clostridium* and *Pediococcus* as indicators to screen the bacteriocin producing organisms. *Lactococcus lactis* ssp. lactis MC38 showed activity against all the gram positive bacteria tested but did not show any activity against gram negative bacteria[31]. It also proved that pediocin NV5 had strong antimicrobial efficacy against *L. mesenteroides, L. monocytogenes* and *Enterococcus faecalis*[32].

Bacteriocingenic strain was identified by 16S rRNA sequence analysis. Bacteriocin producing strains were identified on the basis of nucleotide sequence of the 16S rRNA gene and identified as *Lactococcus lactis* subsp. Lactis[33], *E. faecalis*, *Pediococcus acidilactici* and *Pediococcus pentosaceus*[34], *Pediococcus* sp.[35] and *B. subtilis*[36].

The crude protein was purified by ammonium sulfate precipitation and dialyzed against phosphate buffer (pH 6). The dialyzed protein was purified by gel filtration chromatography and then lyophilized. Since most of the bacteriocins have positive charge at neutral pH, the use of cation exchange resin is appropriate for their purification[37]. The result of the quantitative study to evaluate the activity units of the bacteriocin showed that there is no difference in activity unit of bacteriocin among the indicators used. In contrast[20], observed that indicator cultures showed different sensitivity to bacteriocin since the activity units were found to be different for each indicator used *Lactococcus monocytogenes A4 (L. monocytogenes)*, *Lactococcus curvatus* and *Lactococcus seelegeri.* However, our result was coincided with Bhattacharya *et al*[38] and they reported that maximum activity of bacteriocin (12 800AU/mL) was recorded in MRS broth when the pH was adjusted to 5.5, 6.0 or 6.5.

Besides a broad spectrum activity against gram positive and gram negative bacteria *B. cereus* bacteriocin was found to be thermostable and wider pH tolerance. The results showed that the *B. cereus* bacteriocin used in this study showed their activity from acidic to alkaline conditions and its confirmation was not much affected even at pH 3 and 10. So it can be used as an effective preservative for acidic foods. Our results were correlated to the study of Coventry *et al*[19] and reported that the bacteriocin showed maximum activity over a wide range of pH (3-10). In addition loss of activity after the treatment with proteinase K, trypsin and papain confirmed the proteinaceous nature of bacteriocin and this was not affected by other enzymes like amylase, lipase, and diastase suggesting the absence of glycosylated and lipid moieties. It's also found similar results while treating with proteolytic and non proteolytic enzymes[39]. The bacteriocin showed activity till 100 ℃ for 15 min and at pH 7 and it was not activated by trypsin, lipase and catalase[40]. The bacteriocin treated with organic solvents was not shown any change in inhibitory effect[16].

The gram negative bacteria are poorly sensitive to bacteriocin and higher concentration is needed to inhibit their growth. So to increase their activity against Gram negatives the bacteriocin was treated with EDTA, NaL and KS. Bacteriocin-EDTA combination showed better activity against both gram positives and gram negatives. But the Gram negatives are more susceptible to bacteriocin when it was treated with EDTA, NaL and KS. The EDTA treated bacteriocin showed better result than NaL and KS. Boziaris *et al*[41] showed that treatment with nisin and chelators reduce the population of gram negatives. This is coincided with other's report[42] and they found that only EDTA and pyrophosphates were able to cause appreciable inhibition of *E. coli* by nisin. The present *in vitro* study indicates that the EDTA could be used with bacteriocin to increase the antimicrobial activity against Gram negatives. The combination treatments with nisin-NaL, nisin-KS, NaL-KS and nisin NaL-KS but not nisin-EDTA gave significant reductions of *Salmonella* directly inoculated onto fresh cut piece of Cantaloupe[23]. The antimicrobial activity of lactic acid is not only due to lowering the pH and also due to disrupt the outer membrane of gram negatives. The inhibition of gram negative bacteria by EDTA is due to chelation of divalent cations found in the cell wall[43]. KS (0.02%-0.30%) is widely used in food to inhibit yeast, mold and bacteria[44]. As these three are considered as GRAS we may use these three along with antimicrobial peptide, bacteriocin to control the microbial growth in food system.

The mode of action of bacteriocin was studied against *L. monocytogenes* and it proved that bacteriocin showed a bactericidal action and that was found to be concentration dependent. The bactericidal effect was proved within 1h. It

is generally accepted that bacteriocin induced cell death is found to be a concentration and time dependent process[1]. The death is due to the increased permeability of the cytoplasmic membrane of the target cells, which leads to the release of small cytoplasmic particles and depolarization of membrane potential[30]. The bacteriocin produced by *B. cereus* was found to be low molecular weight protein. This result is agreement with those obtained from the SDS- PAGE of bacteriocins produced by *B. cereus*[45], *B. megateirum*[46] where low molecular weights were estimated to range from 4-6 KDa and 3.496 to 6.512 KDa. Similarly, Teixeria *et al*[47] reported that bacteriocin of *B. circulans* had an approximate molecular weight of 3.5KDa. FTIR analysis of bacteriocin stated that it may be containing aliphatic C-H stretching of fatty acids and aromatic hydrocarbons and the amide Ⅰ and amide Ⅱ is corresponding to the presence of protein. The physical, chemical and biological characterization of *B. cereus* bacteriocin revealed interesting properties that proves its importance regarding food safety. It has impact on its efficacy in the control of food spoilage microorganisms.

Conflict of interest statement

We declare that we have no conflict of interest.

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