Bacteriocin Production in *Bacillus cereus* Food Isolates with Molecular Detection of *cerA* gene

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Abstract

Sixty-three *Bacillus cereus* strains isolated from food samples. All strains were subjected to DNA sequencing for 16S rRNA for identification. 15 strains were registered at GenBank of NCBI and given new accession numbers. 41.26% of the isolates showed bacteriocinogenicproduction activity against four bacterial speciesviz,*Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp. Presence of *cerA* gene coding for cericidine(a type of bacteriocin) was detected in 7.69% of the isolates that produced bacteriocin. Bacterionwas precipitated by ammonium sulphate and purified by dialysis. Antimicrobial activity of precipitated bacteriocin against the four types of bacteria showed the effect on *Bacillus cereus* and *Staphylococcus aureus* growth but not on *Escherichia coli* and *Salmonella* spp.The produced bacteriocin has a molecular weight ranging from 47-54 KDa.Estimation of the concentration and physical and chemical properties of bacteriocin were also investigated.

***Key word**: Bacteriocin, Bacillus cereus, cerA, 16SrRNA.*

Introduction

Bacteriocins are bacterial products which acts as antimicrobial peptides. They are ribosomally synthesized and secreted to act closely related to one another of bacterial species. Bacteriocins are used as antimicrobials for the treatment of human and animal infections. Such products will minimize the increased bacterial resistance to conventional antibiotics. In addition, since consumers require minimally processed foods without chemicals, natural antimicrobial research such as bacteriocin has increased[1]. Bacteriocins have an antimicrobial action affecting cell wall or cell membrane[2]. Bacteriocins are low molecular weight polypeptides with heat stable and proteolytic enzyme sensitivity[3,4]. Using of food additives has been reduced due to safety concerns. Chemical additives are sometimes replaced by the use of natural products of microflora using their antimicrobial activity to increase the lifespan and safety of foods[5,6].This study aims to isolation and purification of bacteriocin from *Bacillus cereus* and determination the molecular weight by SDS-Page. In addition to study its antimicrobial activity against several type of medically important bacteria.

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Materials and Methods

Bacterial strains

Bacillus cereus strain was isolated from food samples from a previous study[7]. 63 of *B. cereus* strains were further identified by 16S rRNA partial sequence. Genomic DNA was extracted from bacterial cells cultured using a commercial kit provided by the manufacturer (Geneaid). Extracted DNA were stored at -20°C until used. The 16S rRNA oligonucleotide primers which were used have 1541 bp and their sequence are: forward, AGAATTTGATCCTGGCTTAG and reverse, AAGGAGGTGATCCAGCC[8].

Screening of bacteriocin production in the isolates

Cross-streaking antimicrobial activity assay

Bacillus cereus strain was grown on Brain heart infusion agar supplemented with 1.5 % glucose. For testing the antimicrobial activity of*B. cereus*, strains were cross-streaked at 24 hrs pre-incubation. A sterile loop was streaked vertically in the center of BHIAG plate to form a single line, incubated at 35° C for 24 hour[9,10]. Four species of bacteria used such as *B. cereus, S. aureus, E. coli*, and *Salmonella* sp. The types of bacteria cultured horizontally on *B. cereus* from the edge to the center, after 24 hour of incubation, a clear of inhibition region was appeared, and this referred to bacteriocin production[11].

Molecular detection of *cerA* gene in *Bacillus cereus* isolates

The *cerA* gene was detected using theprimers with product size 233bp. The pair of primer used wasforward: ATGTCAAAAGGATACAAGTTCACAA and revers: TTATTTACAAATCTTAATTGACGT[12].The PCR tubes were transferred to the thermolcycler to start the amplification reaction according to the specific program.The PCRtemperature conditions were 94°C denaturation, 55°C for annealing step and 72°C for extension step. PCR products were detected in 1.5 % agarose gel stained with ethidium bromide and viewed by U.V. transilluminator.

Production of bacteriocin

The bacteriocin producing bacteria were grown in Brain-Heart Infusion broth (BHIB) supplemented with 1.5 % glucose[13]. After 18 hrs. incubation, the fermented broth was centrifuged at 8500 rpm for 20 mins[14].The supernatant was precipitated with 60% ammonium sulfate (w/v) and left tosettle overnight. To collect the precipitate, the product was cooled centrifuged at 5000 rpm for 45 min. The precipitate 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°C for overnight[15]. The produced pellets were centrifuged anddissolved inamount of water. 0.1 ml of the solution was tested for the antibacterial activity using well diffusion assay[14]. The protein then detected by biuret test using biuret reagent[17,18].

Estimation of extracted bacteriocin concentration

The concentration of extracted protein was estimated by using a spectrophotometer and a wavelength of 280 and 260 nm depending on the following equation[19];

Concentration of protein g / ml = 1.55 × A 280 -0.77 × A260, Where A = absorbency

Determination of bacteriocin activity at different conditions

The effect of pH[20], temperature[21], proteinase K and lysozyme[22], and EDTA[23]on purified bacteriocin activity against bacteria were estimated.

Determination of bacteriocin molecular weight

This was estimated by electrolysis by using a poly-acrylamide gel[24,25].

Antimicrobial activity of bacteriocin

The antibiotic susceptibility testing was done by the well diffusion method[26]. The wells were prepared using sterile yellow tips and filled with 100 µl of extracted bacteriocin. The plates were placed in an incubator for 18hours at 37 ºC.

Results

Identification of studied bacteria

All bacterialisolates identified by 16S rRNA showed the similarity of 99% when it blasts in the NCBI database. 15 isolates showed mutation change at different loci were registered at GenBank of NCBI and given the following accession numbers (Table 1).

Screening the bacteriocinogenic isolates

Out of 63 *B. cereus* used in this study, 26 (41.26%) strains showed bacteriocin production activity. Screening of bacteriocine was done against four bacterial species. Some of the isolates showed maximum inhibition activity.

Table 1. Strains of *Bacillus cereus* that registered at GenBank and their accession numbers.

No. Of sample	Nucleotide change	Accession number	No. Of sample	Nucleotide	Accession number
BBS1	T>G, G>C	MK468691	BBS8	T>G,G>C,T>C	MK468736
BBS2	G>A,T>G	MK468692	BBS9	T>G	MK468798
BBS3	G>C,G>C	MK468693	BBS10	T>G	MK471340
BBS4	G>A	MK468700	BBS11	G>A	MK468901
BBS5	T>G	MK468704	BBS12	T>G	MK468902
BBS6	T>G,A>C	MK468727	BBS	G>C,G>C,G>C,G>T	MK480518
BBS7	C>T,G>A,T>C,G>C	MK468732	BBS13	C>T ,A>T, A>C, C>T, A>C, C>T, C>G, C>T, C>G, C>T	MK949281

Molecular detection of bacteriocin Cerecidin (*cer A)* gene

The bacteriocin cericidine A gene was found in 2 isolates (7.69%) out of 26 isolates as indicated in figure (1).

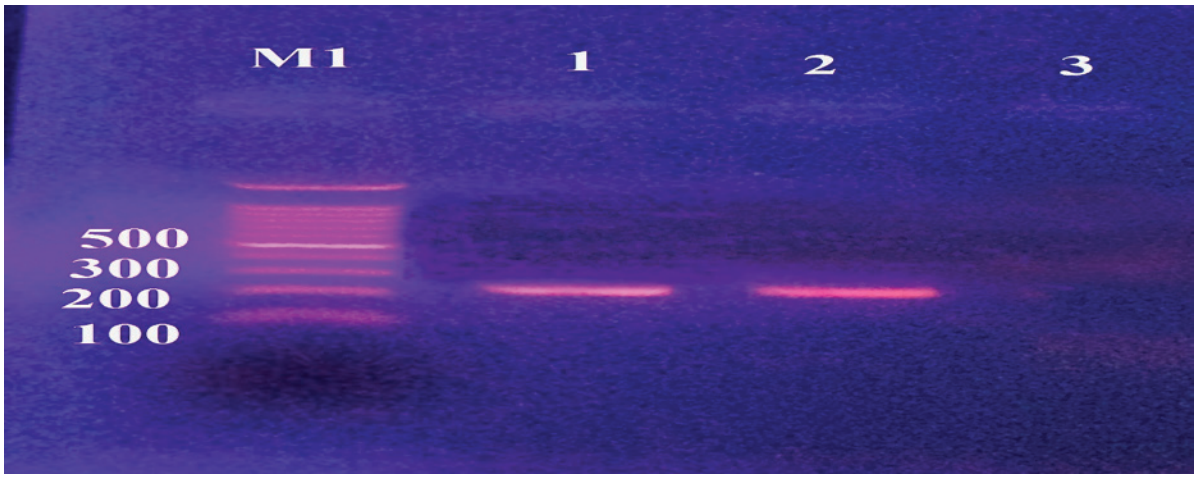

Figure 1. Detection of bacteriocin *cerA* gene by PCR .Lane M1= molecular marker; Lane 1-2=Positive for Cerecidin*cer A* gene approximately 233 bp; Lane 3= negative for Cerecidin*cer A* gene

Biological activity of crude bacteriocin

The crude bacteriocin was produced by *Bacillus cereus*cultured grown in BHIB for 18-24 hrs. Using ammonium sulfate and dialysis.The biological activity of crude bacteriocin was tested against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* spp. The results showed activity against *Bacillus cereus* and *Staphylococcus aureus* but no activity was detected against *Escherichia coli* and *Salmonella* spp. Table (2).

Table 2. Antimicrobial activity of crude bacteriocin against different types of bacteria

Types of bacteria	Inhibition zone (mm)
Bacillus cereus	10 mm
Staphylococcus aureus	6 mm
Salmonella spp.	0 mm
E. coli.	0 mm

Estimation of extracted bacteriocin concentration

The results show that the concentrations were ranged between 217.308 to 648.606 (Table 3).

Table 3. Estimation of bacteriocin concentration

No. of samples	260 nm	280 nm	Bacteriocin conc. gm/ml
Crude	1	0.962	217.308
1	2.570	2.680	648.606
2	1.986	2.290	596.2628
3	2.860	2.999	555.828
4	1.972	2.060	461.0536

Physical and chemical properties of bacteriocin

Thebacteriocin was active in a range from pH 3-11, with a best antimicrobial activity at pH 7 when tested on Gram-positive bacteria, *B. cereus* and *staph aureus*.

The bacteriocin was active at 30 °C on *B. cereus*,and *S. aureus* and lost its activity at high-temperature Table (4).

Table 4. The effect of different pH on bacteriocin

Isolates	pH			Temperature			
	pH 3	pH7	pH 11	30 °C	50 °C	70 °C	90° C
B. cereus	6 mm	11 mm	5 mm	+	-	-	-
S. aureus	6 mm	8 mm	5 mm	+	+	+	-

The bacteriocin lost its activity on *B. cereus* and *S. aureus* when treated with proteinase K and lysozyme.The addition of EDTA increases the activity of bacteriocin. The results showed high activity against *B. cereus* and *S. aureus* and Gram-negative *E. coli* and *Salmonella* spp. Table.

Estimation of the molecular weight of bacteriocin

The molecular weight of the protein was estimated by electrolysis by using a poly-acrylamide gel according to Lammeli method**.** The estimated molecular weight of bacteriocin was 47-54 KDa.

Discussion

*Bacillus cereus*produced a peptide that showed antimicrobial activity against major food-borne bacteria[27].Our results suggest that this substance has a bactericidal effect against *B. cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* spp. by inhibition the growth of these bacteria. The effect may depend particular test conditions, such as the quantity and purity of the bacteriocin, the indicator strain and concentration of cells[28].

In this study, the bacteriocin was purifiedand the activity of this crude bacteriocin appears against Gram-positive bacteria 10 mm and 6 mm for *B. cereus* and *S. aureus,*respectively. There was no activity on Gram-negative bacteria. In this study, thebacteriocin was active in a different pH value, but the maximum activity was found at pH 7 when tested on Gram-positive bacteria, *B. cereus* and *staph aureus*. The stability of bacteriocins at various pH scales is a limiting factor for their use in food[29].In this study, the activity of bacteriocin was lost when the temperature was increased. This was agreed with Sankar*et. al.,*[29].

In vitro, the present study shows that EDTA with bacteriocin used against Gram-negative bacteria to boost antimicrobial activity[30]. The gram-negative bacteria are poorly sensitive to bacteriocin and require increased concentration to inhibit growth. The bacteriocin was treated with EDTA to increase activity against Gram negative bacteria. The combination of bacteriocin and EDTA showed better antimicrobial activity. The bacteriocin lost its activity on *B. cereus* and *S. aureus* when treated with proteinase K and lysozyme, no inhibition zones are formed[30].In this study, single band of the purified bacteriocin appeared in SDS-PAGE. The product has a molecular weight ranging from 47-53 kDa, whereas in another study, the bacteriocin determined by SDS PAGE is 23 kDa[22]. The bacteriocin produced by *Bacillus cereus* GN105 showed a bacteriocin band at 3.5 kDa[31], at21 kDa[32]. and 43 kDa[12].

Conclusion

Several studies were done on *Bacillus cereus*in the similar area of study[33,34,35,36,37]. Bacteriocin production rarely investigated which considered as effective food preservatives. Though cerecidine A is the only the detected bacteriocin in this study. Bacteriocins have inhibitory action against food-borne pathogens such as *Bacillus cereus* and *Staphylococcus aureus*. The action of bacteriocinincreased by combining with EDTA.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: Self-funding

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