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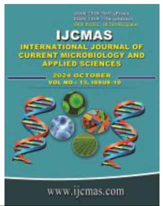

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Screening of Native *Bacillus thuringiensis* Strains for Bacteriocin Production and Characterization of Bacteriocin and Anticancer Activities of *Bt28Q1* Strain

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ABSTRACT

Keywords

Bacillus thuringiensis, bacteriocin, partial purification, SDS-PAGE, Gel Overlay, anticancer

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Bacillus thuringiensis (*Bt*) is a main producer of insecticidal proteins but it has also a good potential to produce antibacterial proteins like bacteriocins. In this study, the bacteriocin production potential of native *Bt* strains in our collection were investigated. The antimicrobial activity of 100 *Bt* strains were screened by agar spot assay and 17 of them with clear inhibition zones were further analysed by well diffusion assay. Among them, *Bt*-28Q1 strain resulted in a highest inhibition zone against *Bacillus cereus.* The antimicrobial substance was detected at the highest level at the beginning of logarithmic phase in the growth curve. Its production was at the maximum level in Tryptic soy broth (TSB) compared to other medium. The antimicrobial substance was heat-stable, pH-stable (range 5-9), and proteinase K sensitive, similar to bacteriocins. This bacteriocin was partially purified with ammonium sulfate precipitation and its molecular weight was determined as around 12 kDa via SDS-PAGE and gel overlay assays. Bacteriocin showed antimicrobial activity to most of the *Bacillus* species and anticancer activity against human cancer cell lines A549 and HT-29. Sequence analysis of *16S rDNA* and flagellin *hag* genes of the *Bt* 28Q1 strain identified it as *Bacillus thuringiensis* subsp. t*ochigiensis.* These findings indicate that bacteriocin from *Bt*-28Q1 has potency for being used as bioactive molecule to prevent the growth of food spoilage bacteria and for development of a new anticancer agent.

Introduction

Antibiotics have a great importance to combat against microbial pathogens. However, an intensive use of antibiotics led to emergence of resistance to almost all current antimicrobial drugs so far (Sumi *et al.,* 2015). Therefore, discovery of new antibacterial agents became necessary to fight against antimicrobial resistance (Coates *et al.,* 2002). Among these, antimicrobial peptides (AMPs) can be a promising alternative to traditional antibiotics. AMPs kill the microbes by opening pores in the membrane and make emergence of resistance more difficult (Sumi *et al.,* 2015). However, conventional antibiotics target metabolic enzymes that play a role in the development of resistance.

Bacteriocins are ribosomally synthesized anti-microbial peptides with a length of 20-60 amino acids (Kumariya *et*

al., 2019). These AMPs cause no harm on the host bacterium because of posttranscriptional modification and/or specific immunity mechanisms (Hancock and Chapple, 1999). On the other hand, AMPs are active against either in the same species and called narrow spectrum bacteriocins or in bacteria from different genera (Maróti Gergely *et al.,* 2011). Since bacteriocins have a high potency and specificity, they have being used as potential antibacterial agents for many applications, including food preservation and infection treatment (Hassan *et al.,* 2012). For example, nisin produced by lactic acid bacteria (LAB) has long been used in food preservation (E234) (Gharsallaoui *et al.,* 2016). In addition, bacteriocins have shown cytotoxic activity against cancer cells, and therefore they could be considered as tools to develop new anticancer drugs (Cesa-Luna *et al.,* 2021).

The genus *Bacillus* has potential to produce a large number of AMPs. Therefore, it seems like a promising source in the search for new inhibitory substances (Xie *et al.,* 2009). *Bt* is well known for synthesizing variety of compounds with biological activity. Although *Bt* has been well known for its insecticidal proteins, it is also a good potential source in the production of bacteriocins. So far, 18 bacteriocins synthesized by different strains of *Bt* have been reported (Sumi *et al.,* 2015; Salazar-Marroquín *et al.,* 2016). These bacteriocins are produced by several subspecies, including *morrisoni*, *kurstaki*, *kenyae*, *entomocidus*, *tolworthi*, and *tochigiensis* (De la Fuente-Salcido *et al.,* 2013). The evidence from the literature indicate that different *Bt* strains are very attractive alternatives for obtaining new active bacteriocins. Therefore, the aim of this study is to investigate the potential to produce bacteriocin-like substances of approximately 100 *Bt* strains in our *Bt* collection and to characterize one of these strains in more detail.

Materials and Methods

Bacterial Strains

In this study, *B. cereus* 11778 ATCC was used as an indicator strain. *Bt* subsp. *kurstaki*, *Bt* subsp. *aizawai*, *Bt* subsp. *tenebrionis*, *Bt* subsp. *israilensis*, *Bt* subsp. *dacota* were obtained from Bacillus Genetic Stock Center (Ohio, USA). *Bt* subsp. *finitimus, Bt* subsp. *galleriae*, *Bt* subsp. *thompsoni*, *Bt* subsp. *morrisoni* were supplied from Ege University, Department of Microbiology. *Listeria spp.* and *Candida albicans* were kindly provided by Gamze Basbulbul from ADU. The other bacterial strains used for antimicrobial assays were *Escherichia coli* (ATCC 11230), *Pseudomonas aeruginosa* (ATCC 29212), and three Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538/P), *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (NRRLB-4375).

Determination of Antimicrobial Activity by Agar Spot Assay

The antibacterial activity of 100 *Bt* strains against *B. cereus* 11778 ATCC was initially tested by agar spot assay (Barboza-Corona *et al.,* 2007). Briefly, *Bt* strains were inoculated to TSA and incubated overnight at 28°C. A single colony was transferred to 5 mL of Tryptic Soy Broth and incubated at 28 °C for 4-5 hours. The 2 µl of culture was spotted on TSA and then incubated at 28 °C overnight. The indicator strain *B. cereus* was cultured in TSB until it reached to an OD_{600} of 0.2-0.3 (1×10^9 cells mL^{-1}), at that point 100 µl of *B. cereus* culture was transferred to soft agar (TSA 0.7% wt/vol). The 10 mL of soft agar containing the indicator strain was poured onto the *Bt* cultures on TSA petri dishes. After overnight incubation at 28 °C, the inhibition zones around the *Bt* culture spots were measured.

Agar Well Diffusion Assay

The 17 *Bt* strains exhibiting the clear inhibition zones after the agar spot test were used for well diffusion assays as previously described (Rogers and Montville, 1991; Izquierdo *et al.,* 2008). After culturing in 3 mL of TSB broth for 24 hours, *Bt* cultures were centrifuged at 8000 *g* for 10 minutes at 4 °C.

The culture supernatants were filtered with a 0.45 µm pore size membrane and stored at -20 °C. Besides, *B. cereus* culture was prepared and the 25 mL mixture of soft agar and 50 µl of indicator strain *B. cereus* culture (1×10^9 cells mL^{-1}) was poured into sterile petri dishes. After the soft agar became solidified, the wells were opened with a sterile tip that has a diameter of 6 mm. Then, 90 µl of CFS was put into the wells and allowed to diffuse for 22 h at 4°C. After that, the plate was incubated at 28°C for 24 hours and the diameters of the zones were measured.

Kinetics of Bacteriocin Production

The selected *Bt* strain 28Q1 was cultured in TSB at 28°C for 2-4 hours until the OD at 600 nm reached to 0.1. The

250 µl of preculture was inoculated to 100 mL of TSB medium and incubated for 72 hours at 28 °C. During the incubation, 2 mL of *Bt* culture was taken every 4 hours and 1 mL of that was used for the absorbance (OD_{600}) measurement and the other 1 mL for agar well diffusion test. Every *Bt* culture was centrifuged at 8000 *g* for 10 minutes at 4˚C.

After that, supernatant was filtered and the 2-fold serial dilutions (1, 1/2, 1/4, 1/8, 1/16, 1/32) were prepared. The 90 µl of each dilution was added to the wells of soft TSB petri dishes containing the indicator bacteria *B. cereus*.

After keeping at 4˚C for 22 hours for diffusion, it was incubated at 28˚C for 24 hours. Bacteriocin activity was determined as AU mL^{-1} by multiplying the dilution at the last zone with 1000 and dividing by 90 µl.

Characterization of Bacteriocin

B. thuringiensis strain was grown in TSB at 28˚C for 8 hours and the culture was centrifuged at 8000 *g* for 10 minutes at 4˚C. Then, the cell free supernatant (CFS) was filtered and used to investigate the effects of various temperatures, pH levels, enzymes, organic solvents and media on bacteriocin activity.

After treatment with those different conditions, the CFS were two fold diluted (1, 1/2, 1/4, 1/8, 1/16, 1/32) and the agar well diffusion assay was applied to determine bacteriocin activity against *B. cereus*.

Cell free supernatant was incubated at different temperatures (30, 50, 70, 100 ˚C) for either 30 minutes or 2 h. Then, samples were cooled. The pH values of CFS was adjusted to the pH 3, 4, 5 and 6 with HCl; and to pH 7, 8, 9 and 10 with NaOH. After 30 min incubation at room temperature, each pH value was neutralized. Supernatant was treated for 2 hours with each enzyme: proteinase K, lipase, trypsin, or α-amylase at a final concentration of 1 mg mL^{-1}.

The CFS was treated with each of organic solvent (10%) for 1 hour at room temperature. Samples from all treatment above were assayed for bacteriocin activity. Finally, *Bt* 28Q1 strain was incubated in Tryptic soy broth (TSB), Nutrient broth (NB) and Muller Hinton broth (MHB) for 8, 16, 24 hours at 28 ˚C. Then, CFS was obtained and tested for bacteriocin activity in order to determine how different media effect bacteriocin production.

Partial Purification of Bacteriocin

The *Bt*-28Q1 strain was grown at 28°C in 100 mL of TSB for 8 hours based on bacteriocin production kinetics. The CFS was obtained by centrifugation of the bacterial culture at 8000 *g* for 10 minutes at 4°C. Then, solid ammonium sulfate was added to 60%, 70% and 80% saturation at 4°C with constant stirring overnight. Next, precipitated proteins were centrifuged at 8000 *g* for 30 min at 4°C and resuspended in an appropriate amount of 20 mM Tris-HCL (pH 7) buffer.

Then, this sample was dialyzed against the same buffer at 4˚C for 6 hours in the mini dialysis tubing (SnakeSkin™ Dialysis) with molecular weight cut-off 10000. The dialyzed bacteriocin was filtered through a 0.45 µm filter. Then, it was concentrated by ultrafiltration with vivaspin 500 (Sartorius), aliquoted and stored at -20 ˚C. The protein concentration of sample was determined by Bradford assay.

Antimicrobial Spectrum of Partialy Purified Bacteriocin

The partially purified bacteriocin obtained from 80% ammonium sulfate precipitation was tested against 16 Gram (+) bacteria, 2 Gram (-) bacteria and 1 fungus by the agar well diffusion assay. Muller Hinton broth containing 25 mL of soft agar (0.7%) was mixed with indicator bacteria or fungus adjusted to 0.5 McFarland standart. It was poured into a petri dish and then wells were opened. The 90 µl of bacteriocin was added to the wells and incubated at 28 °C for 24 hours. *B. cereus* indicator strain served as positive control.

SDS-PAGE and Gel Overlay Assay

Partially purified bacteriocin was concentrated using an ultra filtration membrane (Vivaspin 500, Sartorius) with a size of 10000 MWCO. The protein profile of concentrated bacteriocin was analysed by 15% SDS-PAGE gel electrophoresis (Laemmli, 1970).

Bacteriocin sample was boiled for 5 minutes for molecular weight analysis; however, unboiled sample was used for the gel overlay assay. Also, sample loading buffer did not include SDS. One of the gels was stained with Comassie Brilliant Blue R-250. The other gel was fixed with 25% isopropanol and 10% acetic acid for 30 minutes. After that, the gel was repeatedly rinsed with

ddH_2O overnight at 4 ˚C and then placed in a sterile petri dish. TSB soft agar (0.7%) containing *B. cereus* (0.5 McFarland) was poured on the gel. Then, the plate was incubated at 28°C for 24 hours and inhibition zone formation was examined.

Detection of 16S rDNA and *hag* Gene

DNA was isolated and the primers for *16S rDNA* as well as *hag* gene were selected as previously described (Lopez de la Cruz *et al.,* 2018; León-Galván *et al.,* 2009). Dream Taq PCR master mix (Thermo) was used for PCR reactions. The each reaction contained 12.5 µl master mix, 0.5 µl forward primer (10 mM), 0.5 µl reverse primer (10 mM) and 3 µl of DNA in a total of 25 µl reaction volume. The PCR program for the *16S rDNA* gene was performed as following: initial denaturation 5 min at 95˚C, denaturation 1 min at 95˚C, annealing 1 min at 60˚C, elongation 2 min at 72˚C; final elongation 10 min at 72 ˚C. It was amplified for 30 cycles.

The PCR program for the *hag* gene was performed as following: initial denaturation 5 min at 95˚C, denaturation 1 min at 95˚C, annealing 2 min at 48˚C, elongation 2 min at 72˚C; final elongation 7 min at 72˚C. It was amplified for 30 cycles. The DNA from *Bt* subsp. *kurstaki* (4D1) was used as positive control. PCR products were run on a 1% agarose gel for 30 minutes at 100 volts and visualized with the gel documentation system (Vilber Lourmat). DNA was sequenced by Macrogen and the results were analyzed using BioEdit and BLAST program from NCBI.

Cytotoxicity Assay

Cytotoxic effect of CFS of *Bt*-28Q1 on human colorectal adenocarsinoma (HT-29), lung carcinoma (A549), and normal colon cell line CCd18Co was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide) assay. In this assay, cell viability is reflected by reduction of yellow soluable MTT to insoluble blue formazan crystals by mitochondrial dehydrogenase. A total of 4×10^3 cells/well were seeded in 96-well plates (TPP, Switzerland) in triplicate and incubated for 24 h. Cell free supernatant was added to the wells at 6 different concentrations (2-fold dilution) and followed by incubation for 24, 48 and 72 h. After adding 10 µl of 5 mg mL^{-1} MTT reagent (Applichem, USA) into each well, the plate was incubated for 4 h. Then, the medium was discarded and 100 µl of DMSO was added to each well to dissolve the formazan blue crystals formed in the cells. The absorbance of each well was measured at 540 nm using a microplate reader (Thermo Scientific, Multiskan FC, USA). Cytotoxic effects were determined by comparing the optical density of treated cells against that of untreated cells.

Statistical Analysis

The data were expressed as the mean ± standard deviation (SD) of three independent experiments multiple comparison using EXCEL PRO 2019-QI Macros, Software. A difference was considered to have significance at *$p<0.05$.

Results and Discussion

Determination of Antimicrobial Activity

A total of 100 *Bt* strains from the *Bt* collection were screened using the agar spot assay to determine antimicrobial activity against *B. cereus* 11778. Thirty five out of the 100 *Bt* strains showed antimicrobial activity with inhibition zones ranging from 2 mm to 27 mm. Among these strains, 17 of them exhibiting very clear inhibition zones (Table 1) were further tested by agar well diffusion assay. Nine of 17 *Bt* strains formed inhibition zones at various diameters. The 28Q1 strain, that showed the maximum inhibition zone, 17.5 mm was selected for further analysis (Figure 1).

Determination of Growth Curve and Bacteriocin Production Kinetics

The growth curve and kinetics of bacteriocin production from 28Q1 strain were investigated by measuring the absorbance and bacteriocin activity at different time points (Figure 2). The bacteriocin production was observed at the beginning of the logarithmic phase (first 3-4 h) and continued until the beginning of the stationary phase (24 h). The highest level of bacteriocin activity (178 AU mL^{-1}) was detected at the late logarithmic phase (8 h).

Effects of Temperature, pH, Enzymes, Organic Solvents and Media on Bacteriocin Activity

Effect of different temperatures was examined for characterization. Bacteriocin activity was unaffected after 30 min exposure to 30-40˚C and 47% activity remained

at 50˚C. However, its activity was completely lost at 60-70˚C (Table 2). After exposure to different pH levels for 30 min, bacteriocin activity from *Bt*-28Q1 was found to be completely stable at pH 5, 6, 7 and 8. The inhibitory activity decreased to 41%, 90% and 72% at pH 4, 9 and 10, respectively but no activity was observed at pH 3 (Table 2).

Bacteriocin activity was completely lost as a result of proteinase K and lipase treatment while the 72% and 97% activity was maintained in trypsin and α-amylase treatment, respectively (Table 2). In addition, organic solvents acetone, ethanol, methanol and DMSO did not affect bacteriocin activity (Table 2). Finally, effect of different media on bacteriocin activity was investigated. *Bt*-28Q1 strain was incubated in TSB, NB and MHB media at 28˚C for 24 hours. The highest bacteriocin activity (178 AU mL^{-1}) was observed after 8 h incubation in TSB broth (Table 2).

Identification of 28Q1 Strain by *16S rDNA* and *hag* Gene Sequence

Based on both *16S rDNA* and *hag* gene sequence analysis, *Bt* 28Q1 strain was identified as *Bacillus thuringiensis* subsp. *tochigiensis* because >99% identity was detected. Flagellin protein is encoded by *hag* gene. This protein is responsible for immunological reaction in H serotyping and it allows identification of subspecies for *Bt*.

Partial Purification of Bacteriocin

Cell free supernatant of *Bt*-28Q1 was partially purified with ammonium sulfate precipitation (60, 70 and 80%) and dialyzed. All precipitated samples resulted in 355 AU mL^{-1} antimicrobial activity against *B. cereus* (Figure 3). After ultrafiltration (10000 MCO), protein concentration was determined as 0.5 µg mL^{-1} by Bradford assay. Samples were aliquoted and stored at -20 ˚C until used.

Antibacterial Spectrum of Bacteriocin

The antibacterial substance bacteriocin was obtained from CFS of *Bt*-28Q1 strain. The agar well diffusion assay was carried out to determine antimicrobial spectrum of bacteriocin against 13 Gram-positive, 2 Gram-negative bacteria and a fungus *C. albicans*. While antimicrobial effect was observed on some Gram-positive bacteria, no antimicrobial and antifungal effect was detected against Gram-negative bacteria and *C. albicans* (Table 3). The highest inhibition zone (23 mm) was observed against *Bt* subsp. *azawai*. In addition, this inhibitory substance was found to have antibacterial activity against an important pathogenic and spoilage microorganism *B. cereus* (Table 3).

SDS- PAGE Analysis of Bacteriocin and Gel Overlay Assay

Partially purified bacteriocin was run in 15% SDS-PAGE (Figure 4A). Gel-overlay assay was performed to determine the protein band showing antimicrobial activity. Compared with the molecular weight standard, the protein band about 12 kDa showed antimicrobial activity (Figure 4B).

Anticancer Activity

Cytotoxicity assay was carried out to determine anticancer potential of bacteriocin on A549, HT-29 and CCd18Co cell lines. A549 cells were treated with 2-fold serially diluted CFS from *Bt*-28Q1. The highest cytotoxicity (79%) was observed with undiluted supernatant but cytotoxicity decreased to 64% at dilution ¼ and no cytotoxicity was observed at more diluted supernatant (Figure 5A). When partially purified bacteriocin was examined for cytotoxic activity on A549 cells, 95% cytotoxicity was observed on the cells treated with 50 µg mL^{-1} bacteriocin and cytotoxicity dropped to 80% with bacteriocin at 3 µg mL^{-1} (Figure 5B). These results indicate that the bacteriocin is highly cytotoxic against A549 cancer cells even at lower concentrations.

Anticancer potency of bacteriocin was tested on HT-29 cancer cells and CCd18Co normal cell lines at different time points. Undiluted supernatant resulted in 71, 72 and 85% cytotoxicity on HT-29 cancer cells at 24, 48 and 72 h, respectively (Figure 6). An increase in cytotoxicity on HT-29 cells was observed even with the more diluted supernatant at 72 h incubation in comparison with 24 and 48 h incubation.

In addition, supernatant was more effective on CCd18Co normal cells compared to HT-29 cancer cells at 24 h incubation. However, cytotoxicity increased 1.4-, 1.3- and 1.1-fold in the first 3 highest concentration on HT-29 cells compared CCd18Co cells at 72 h, indicating the selectivity of bacteriocin on cancer cells (Figure 6).

Table.1 The clear inhibition zones of Bt strains in agar spot assay

Number	*Bt* strain	Inhibition zone (mm)
1	42MY	-
2	13MY	-
3	KH3	9
4	KH58	10
5	85Bp	9
6	BY7	14
7	19Q2	12
8	37Q2	9
9	183Q	9
10	174Q	17
11	90Q1	25
12	36Q2	17
13	5Q	19
14	164QX1	19
15	28Q1	19.5
16	51MY	19
17	*Bt*-KE63-64	27

Figure.1 Inhibition zone of *Bt*-28Q1 compared to other strains in agar well diffusion assay**.**

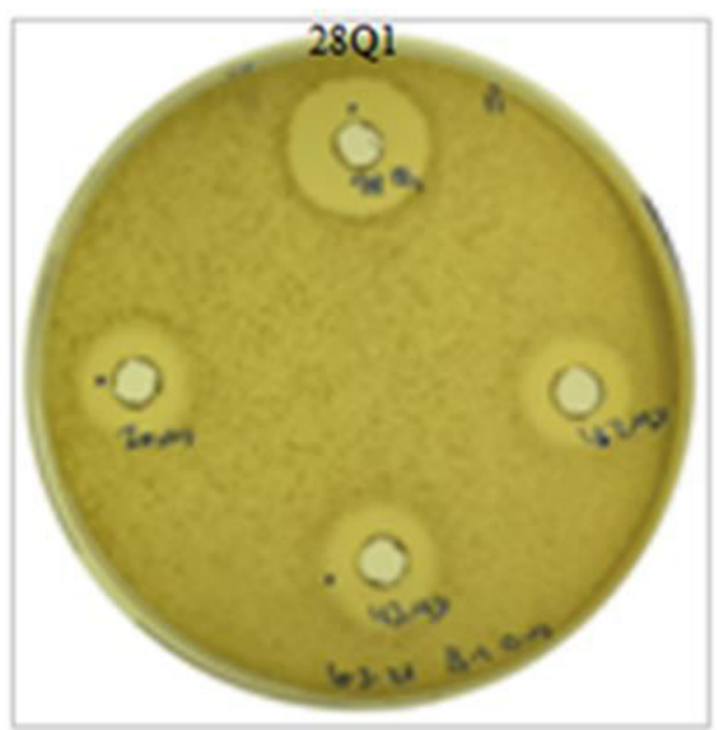


Table.2 Effects of temperature, pH, enzymes, organic solvents and media on bacteriocin activity of supernatant from *Bacillus thuringiensis* 28Q1

Treatment	Time (min)	Residual activity (%)
Temperature (ºC)		
30	30	100
40	30	100
50	30	47
60	30	0
70	30	0
30	120	100
40	120	94
50	120	0
60	120	0
pH		
3	30	0
4	30	41
5	30	98
6	30	100
7	30	100
8	30	98
9	30	90
10	30	72
Enzyme		
Proteinase K	120	0
Lipase	120	0
α-amilase	120	97
Trypsin	120	72
Organic solvents		
Acetone	60	100
Ethanol	60	100
Methanol	60	100
DMSO	60	100
H_2O	60	100
Media		
NB	8h	11 AU mL^{-1}
MHB	8h	22 AU mL^{-1}
TSB	8h	178 AU mL^{-1}

Table.3 Antimicrobial spectrum of supernatant from *Bt* 28Q1 strain

Indicator organisms	Source	Inhibition zone (mm)
Gram positive bacteria		
***Bacillus thuringiensis* subsp. *finitimus* 4B1-4**	BGSC	16
***Bacillus thuringiensis* subsp. *tenebrionis* 4AA1**	BGSC	22
***Bacillus thuringiensis* subsp. *galleriae* 4G1-4G6**	BGSC	22
***Bacillus thuringiensis* subsp. *thompsoni* 4Q1**	BGSC	20
***Bacillus thuringiensis* subsp. *morrisoni* 4K1-4K3**	BGSC	21
***Bacillus thuringiensis* subsp. *dakota* 4R2**	BGSC	18.5
***Bacillus thuringeinsis* subsp. *azawai* 4J3**	BGSC	23
***Bacillus thuringiensis* subsp. *kurstaki* 4D1**	BGSC	18
***Bacillus cereus* 11778**	ATCC	21
Listeria spp	Lab. Isolate	-
Staphylococcus aureus	ATCC	-
Bacillus subtilis	ATCC	-
Micrococcus luteus	NRRLB	-
Gram negative bacteria		
Pseudomonas aeruginosa	ATCC	-
Escherichia coli	ATCC	-
Fungal		
Candida albicans	ATCC	-

Figure.2 The growth curve and bacteriocin production kinetics of *Bt*-28Q1.

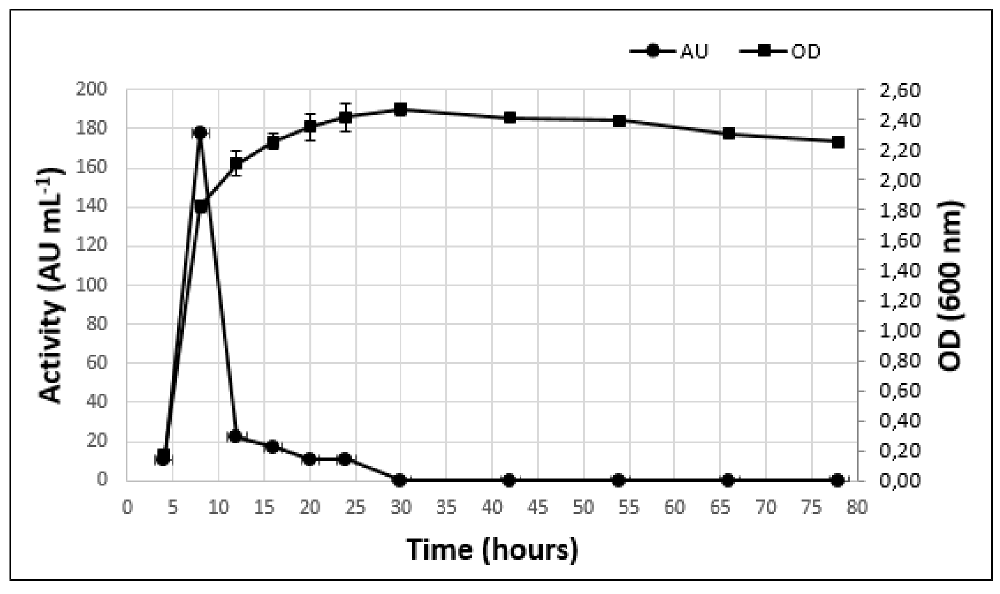


Figure.3 Antimicrobial activity of partially purified bacteriocin against *B. cereus*. Ammonium sulfate precipitation 60% (A), 70% (B), 80% (C).

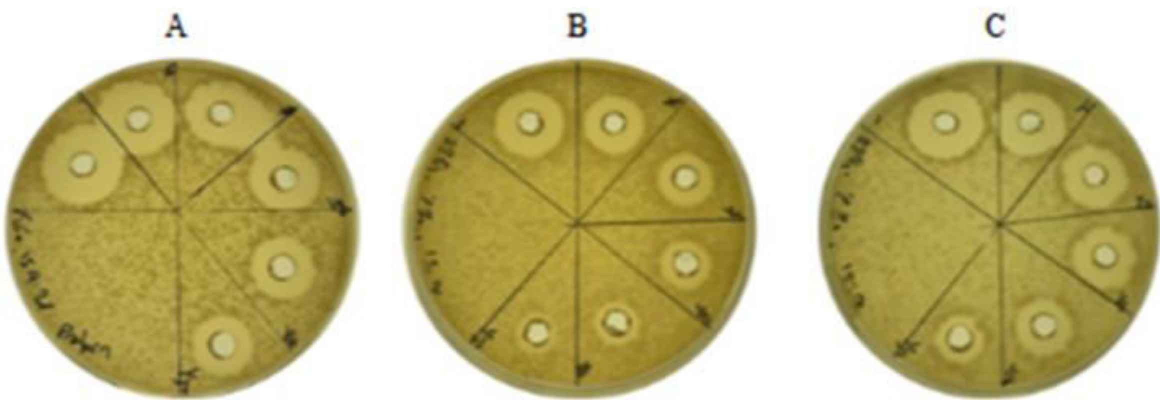


Figure.4 Analysis of protein profile of bacteriocin from Bt-28Q1 strain (A) SDS-PAGE gel electrophoresis of bacteriocin, 1: MW marker (PageRuler SMO661, Fermentas); 2: Protein sample precipitated with 80% AS; 3: Protein sample precipitated with 80% AS and concentrated by ultrafiltration (B) Overlay gel indicating protein band showing antimicrobial activity.

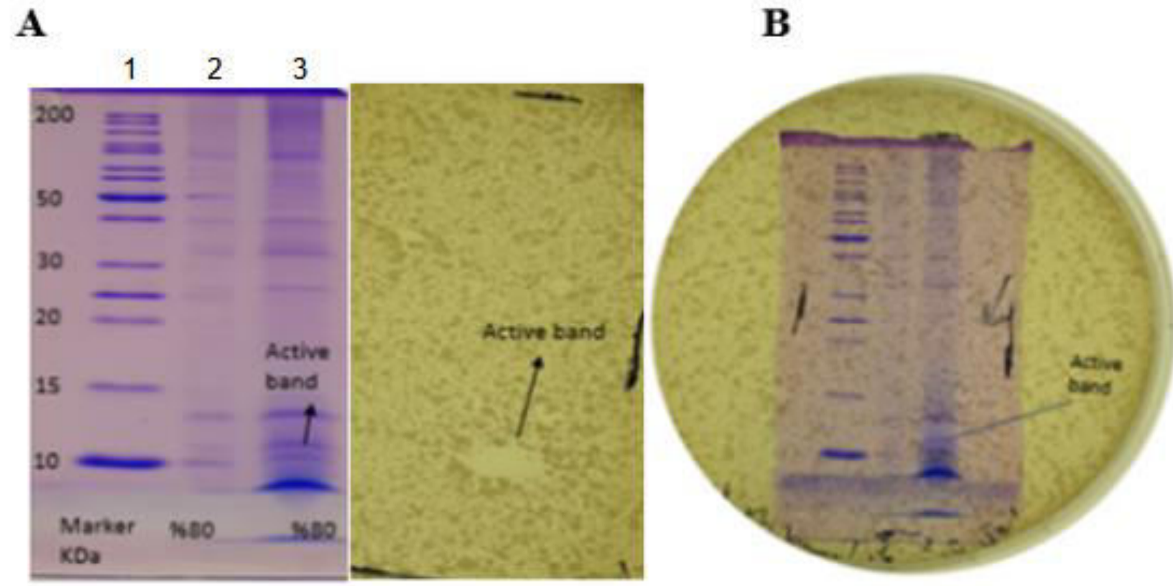
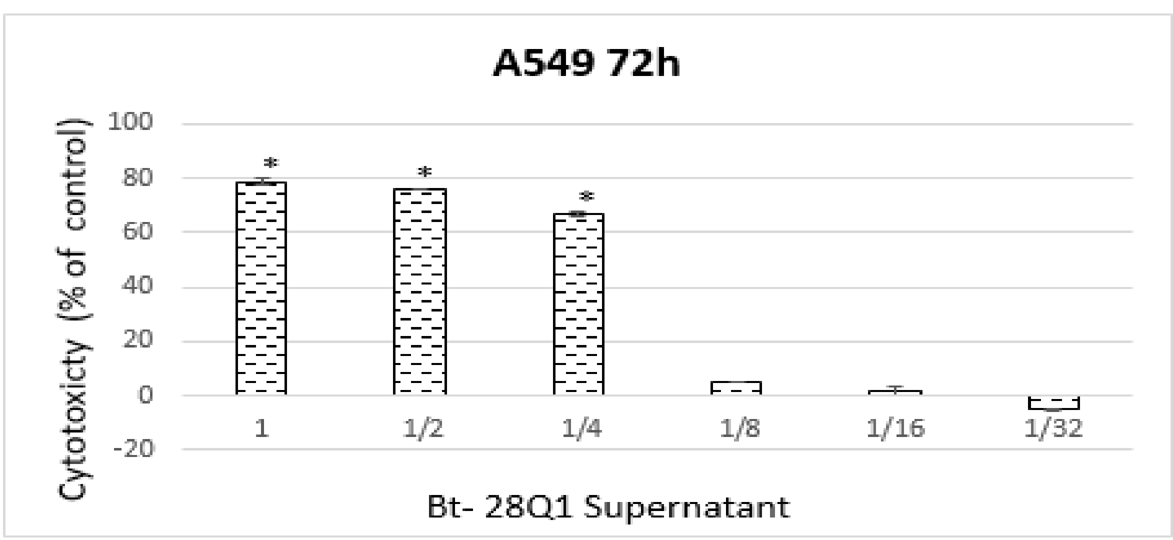


Figure.5 Dose-dependent cytotoxic effects of Bt-28Q1 supernatant (A) and partially purified bacteriocin (B) on A549 cell line. Cells were treated with 2-fold serially diluted supernatant or bacteriocin or left untreated for 72 h. Data expressed as mean ± standard deviation, n=3. *p< 0.05.

A



B

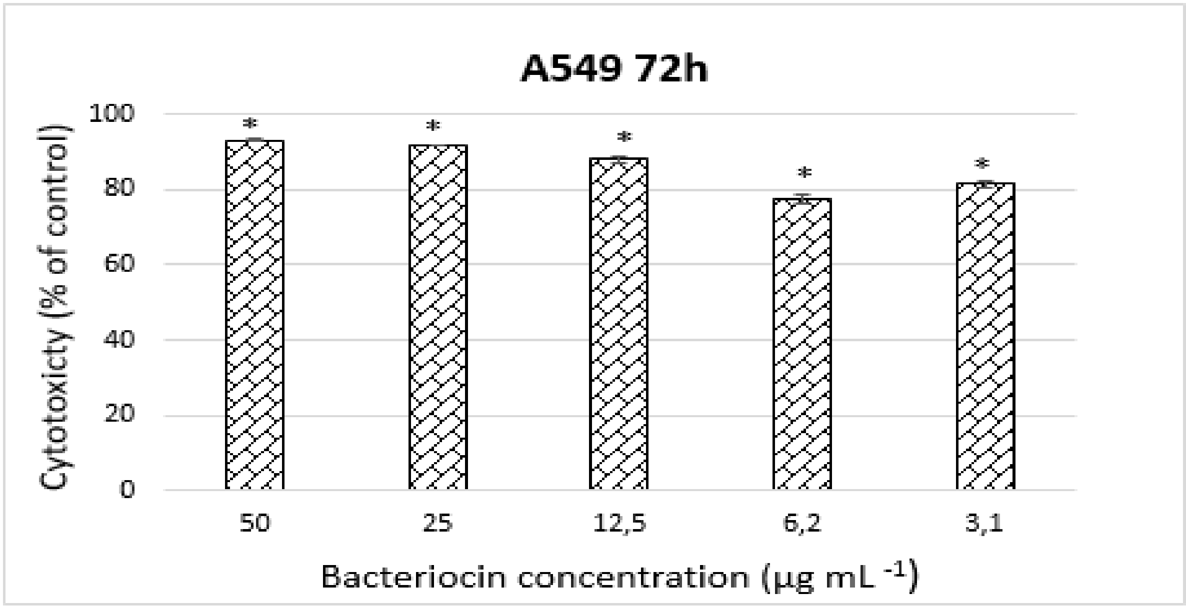
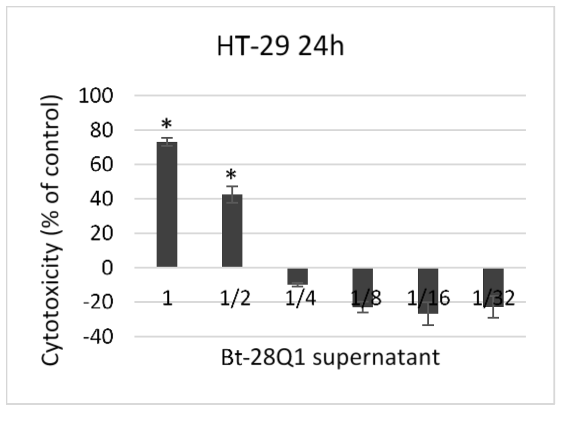
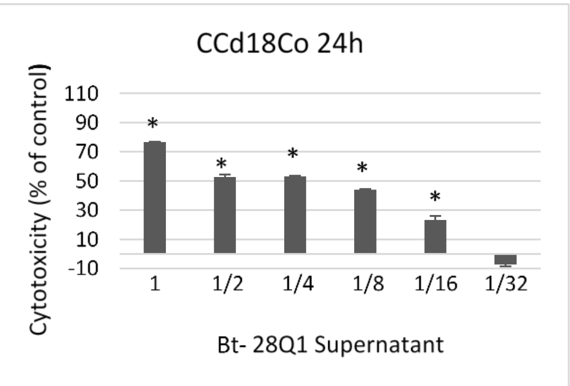
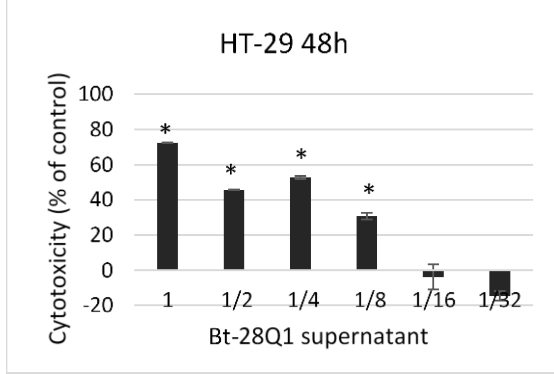
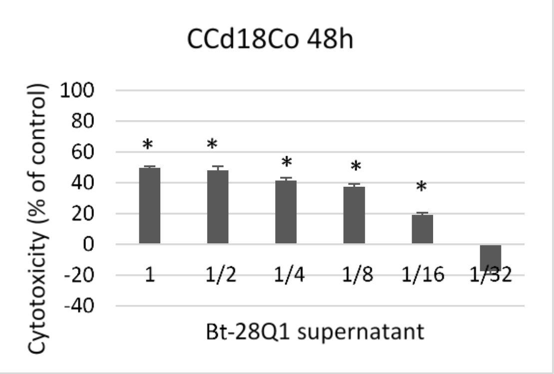


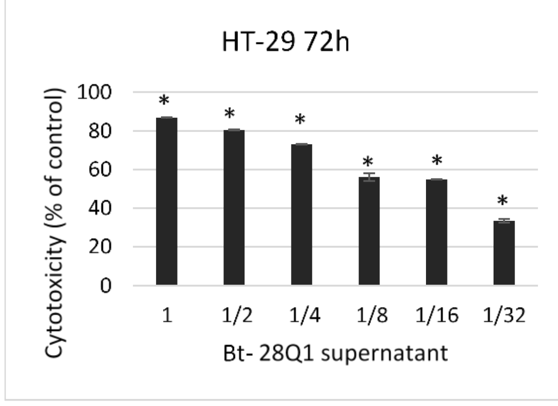
Figure.6 Dose- and time- dependent effects of Bt-28Q1 supernatant on HT-29 and CCd18 cell lines.

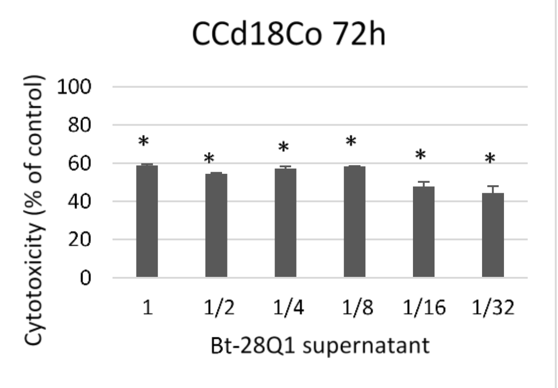












Data expressed as mean ± standard deviation, n=3. *$p < 0.05$.

Bacillus thuringiensis, which is well known with its entomopathogenic properties has also a great potential to produce bacteriocin like antimicrobial peptides. The bacteriocins have been used in food industry for natural food protection (De la Fuente-Salcido *et al.,* 2013; Bode, 2009). Besides, they have been important alternatives to antibiotics in the dealing with the emergence of resistance (Sumi *et al.,* 2015). There has been various research that have investigated bacteriocin like protein preparations of *Bt* strains isolated from different environments (De la Fuente-Salcido *et al.,* 2013). In this study, we searched the potentials of bacteriocin-like antimicrobial activity of 100 *Bt* strains from our collection. First, *Bt* strains were propagated and their antimicrobial activities were screened against the indicator bacterium *B. cereus* (ATCC 11778) by agar spot assay. It was determined that 35 *Bt* strains showed inhibitory activity, and 17 of them formed a very clear

inhibition zones. It was confirmed by the agar well diffusion method that only 9 of 17 strains showed antimicrobial activity against indicator bacterium *B. cereus.*

Because bacteriocins target closely related species that produce them, *B. cereus* was used as an indicator bacterium. This bacterium causes spoilage in food (Barboza-Corona *et al.,* 2007). In this present study, some of the *Bt* strains showed antimicrobial activity in the agar spot assay but not in the agar well diffusion assay. There may be a number of reasons for this. For instance, microorganisms can develop different strategies by competing with each other for nutrients in the environment, and one of these strategies is to produce and secrete antimicrobial substances continuously during the assay.

In addition, the synthesis of bacteriocins can be stimulated when a producer strain recognizes a common substance (inducing factor) present due to a susceptible strain (Compaoré *et al.,* 2013). Compared to flip plate or well procedures, the spot assay method for identifying bacteriocins is more accurate, quicker, and simpler to score (Lewus and Montville, 1991). However, in the spot assay, other metabolites may be included in the inhibitory activity of bacteriocin or comparable metabolites on the examined microorganisms.

To ensure that bacteriocin or related metabolites are the source of antimicrobial action, the agar spot test and agar well diffusion test should be used in tandem (Con and Gökalp, 2000). Therefore, both techniques were used in this work, and the *Bt*-28Q1 strain was chosen for further characterization because it exhibited the largest zone of inhibition (17.5 mm) in the agar well diffusion test.

Bacteriocin production from *Bt*-28Q1 started in the early logarithmic phase and reached its maximum level at the end of the logarithmic phase. Production in the stationary phase has completely ceased. The production of bacteriocin-like peptide in logarithmic phase can be considered as the primary metabolite (Cladera-Olivera *et al.,* 2004). Similar results were obtained in the production of tochisin from *Bt* (Paik *et al.,* 1997), bacteriocin-like peptide from *Bacillus licheniformis* P40 (Cladera-Olivera *et al.,* 2004). It was determined that they started to be produced at the beginning of the logarithmic phase and decreased during the stationary phase. The decrease and subsequent loss of antimicrobial activity may be due to adsorption by producer cells or degradation by extracellular proteases. It has been known that proteases are frequently produced by *Bacillus* species (Bizani and Brandelli, 2002).

Effects of several parameters were analysed on bacteriocin activity. In the case of temperature, the activity decreased to 47% at 50˚C after 30 minutes incubation and it was completely lost when bacteriocin was heated for 2 hours. These findings indicate that the bacteriocin has a relatively low temperature tolerance and it may be reported as heat sensitive bacitracin (Ivanova *et al.,* 1998). The experiments testing the effect of different pH on bacteriocins demonstrated that the bacteriocin from *Bt* 28Q1 was pH-tolerant throughout a wide range (pH 5-9). The activity of CFS from *Bt*-28Q1 strain was not affected by treatments with several organic solvents, similar to the findings of others (Oscáriz and Pisabarro, 2000). Loss of activity with proteinase K and lipase enzymes indicates that the bacteriocin may be a lipoprotein. Loss of activity after lipase may be due to the presence of a lipid fragment involved in antimicrobial activity (Jack *et al.,* 1995; Rattanachaikunsopon and Phumkhachorn, 2006). It is thought that the reason why the activity of *Bt* 28Q1 bacteriocin was not completely lost in the treatment with trypsin may be due to the production of cyclic antimicrobial substances containing unusual amino acids by *Bacillus* species (Cladera-Olivera *et al.,* 2004).

An inhibition zone was clearly observed in the direct detection of antimicrobial activity based on SDS-PAGE and gel-overlay assays. It revealed that the partially purified protein from *Bt*-28Q1 CFS was associated with a single protein band with an apparent molecular mass of about 12 kDa. Also CFS from *Bt*-28Q1 showed narrow spectrum of inhibitory activity only against different *Bacillus* species. No antimicrobial effect was observed on neither other Gram + and Gram - bacteria nor on *C. albicans.*

Anticancer activity of bacteriocin from *Bt*-28Q1 was detected on both HT-29 colorectal adenocarcinoma and A549 lung carcinoma cell lines. Bacteriocin inhibited the proliferation of cancer cells in a dose-dependent manner. Even at the low concentration (3 µg mL^{-1}), it caused 80% cytotoxicity on A549 cells. In addition to that, bacteriocin was found to have selectivity on HT-29 cancer cells compared to normal cell line CCd18. The selectivity may result from the differences between cell membrane of cancer cells and normal cells. It has been known that cancer cells have increased amounts of

negative charge on their outer cell membrane (Dobrzyńska *et al.,* 2005; Hoskin and Ramamoorthy, 2008). Therefore, anticancer effect of the the bacteriocin could be due to its cell membrane permeabilization effect as in the case for other bacteriocins (Sand *et al.,* 2013). In fact, future studies will explain exact molecular mechanism of anticancer activity of the bacteriocin.

Bacteriocin from *Bt*-28Q1 strain exhibited antimicrobial activity only against *Bacillus* species indicating to be narrow spectrum bacteriocin. Thus, it has the potential to be used as a food preservative or bioactive molecule to inhibit the growth of toxin-producing bacteria such as *B. cereus* and to prevent food spoilage. Further, the bacteriocin resulted in selective antiproliferative activity on cancer cell lines compared to normal cell line. Future studies related with protein purification, protein sequence analysis and molecular mechanisms of anticancer activities will allow more detailed characterization of bacteriocin form *Bt*-28Q1.

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Author Contributions

Sarah B.J. Al- Baghdady: Investigation, data curation, writing original draft; Burcu Şahin: Investigation, data curation, writing; Cansu Korkmaz: Data curation and analysis of the data; Hatice Güneş: Conceptualization, data analysis, supervision, Writing the manuscript

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

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