Characterisation and biosafety evaluation of a novel bacteriocin produced by *Bacillus velezensis* BUU004

[1]Butkhot, N., [2]Soodsawaeng, P., [3]Vuthiphandchai, V. and [1,4*]Nimrat, S.

[1]*Environmental Science Program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand*

[2]*Biological Science Program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand*

[3]*Department of Aquatic Science, Faculty of Science, Burapha University, Chon Buri 20131, Thailand*

[4]*Department of Microbiology, Faculty of Science, Burapha University, Chon Buri 20131, Thailand*

Abstract

Development of a novel biopreservative harmless to human health has gained increasing attention to control spoilage and pathogenic bacteria in food products. The aims of the present work were to characterise partially purified substance from *Bacillus velezensis* BUU004 (PBV), evaluate its cytotoxicity, and assess the presence of encoding genes associated with enterotoxin production in *B. velezensis* BUU004. The highest antibacterial activity was achieved at 18 h post-incubation in stationary growth phase. The PBV containing bacteriocin displayed proteinaceous nature because its activity was partially inactivated by chymotrypsin and papain, and fully degraded by pepsin, proteinase K and trypsin. Bacteriocin in PBV was thermoresistant up to 96°C, and pH stable ranging from 3.0 - 9.0. It also showed a broad spectrum of antibacterial activity against *B. cereus*, *B. coagulans*, *Listeria monocytogenes*, *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, *E. coli* O157:H7, and *Salmonella* Typhimurium. The molecular weight of the bacteriocin was approximately 5.75 kDa, and the amino acid sequence revealed 43% similarity with existing antimicrobial peptides as determined by an LC-MS/MS analysis. Furthermore, *B. velezensis* BUU004 produced the bacteriocin that has a low IC_{50} of 927.4 µg/mL to HK-2 cells along with absence of enterotoxin genes: cytotoxin K (*cytK*), hemolysin (*hlyII*), hemolytic enterotoxin HBL (*hblABCD*), non-hemolytic enterotoxin NHE (*nheABC*) and cereulide (*cesABCD*), indicating its basic biosafety characteristic. Our results suggest that a bacteriocin produced by *B. velezensis* BUU004 has promising potential as a safe biopreservative in the food industry.

Introduction

Bacillus velezensis is a member of the *B. subtilis* complex, which includes other closely related species, e.g. *B. amyloliquefaciens*, *B. methylotrophicus*, *B. siamensis*, *B. subtilis*, *B. tequilensis*, *B. vallismortis*, *B. atrophaeus*, *B. mojavensis*, and *B. licheniformis*. Most of them are granted Qualified Presumption of Safety (QPS) approval by European Food Safety Authority (EFSA; Fan *et al.*, 2017) and Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration (Sewalt *et al.*, 2016). Some *Bacillus* species have a long history of safe use in human and animal food productions as well as industrial enzyme and biochemical products

(Harwood *et al.*, 2018). Several species within *B. subtilis* group are also generally considered to be a good producer of versatile secondary metabolites harbouring antimicrobial activity: polyketides, lipopeptides, siderophores, bacteriocins, bacteriocin-like inhibitory substances (BLIS), and non-ribosomally synthesised peptides (Harwood *et al.*, 2018).

Bacteriocins are bacterial peptides synthesised through the ribosomal synthesis pathway and display antibacterial activity against closely-related or non-related bacteria through bactericidal mechanisms such as pore-forming type, nuclease type, and peptidoglycanase type (Yang *et al.*, 2014). BLIS are defined as active antimicrobial peptides and proteins

that lack a well characterisation of their encoding gene, amino acid sequences and biochemical characteristics (Tagg *et al.*, 1976; Abriouel *et al.*, 2011). Bacteriocins and BLIS are proteinaceous compounds with protease sensitivity resulting in easy digestion in gastrointestinal tracts, thereby considering as basically safe food additives (Yang *et al.*, 2014). Because of their antimicrobial activity and no deleterious effects on organoleptic properties of foods, bacteriocins have been used as a preservative in many foods, *e.g.* nisin in kimchi, mashed potatoes, cheese products, milk products, canned products, salad dressings, meat products; pediocin PA-1 in Cheddar cheese, Munster cheese, liquid whole egg, meat products (sausages, meat sticks); and enterocin AS-48 in cider, fruit and vegetable juices, canned vegetables, vegetable sauces, ready-to-eat salads, purees and sausages (Settanni and Corsetti, 2008; Johnson *et al.*, 2018). Currently, bacteriocins and BLIS from *Bacillus* species have increasing importance due to their broader inhibitory spectrum, distinct structural diversity and stability under a wide range of temperature and pH in comparison with most bacteriocin produced by lactic acid bacteria such as nisin (Abriouel *et al.*, 2011).

In accordance with the European Commission Regulation (EFSA, 2014), development of *Bacillus* strains required an assessment of toxigenic potential to ensure the appropriate use in human and animal nutrition either directly as a feed additive or indirectly as a source of such additives. Therefore, a rigid selection approach of bacteriocin-producing *Bacillus* species needs to evaluate their virulent characteristics. In recent study, a semi-purified substance from *B. velezensis* BUU004 was proven to control the growths of foodborne pathogenic bacteria in dried seafood products (Butkhot *et al.*, 2019). In the present work, we characterised a bacteriocin produced by *B. velezensis* BUU004 and determined its cytotoxicity. In addition, we evaluated the presence of toxin genes associated with gastrointestinal illness in *B. velezensis* BUU004 genome.

Materials and methods

Bacterial strain and culture condition

B. velezensis BUU004 was reported to produce antimicrobial substances that had a biopreservative potential and antibacterial property due to wide inhibitory spectrum against foodborne pathogens in crushed seasoned squids (Butkhot *et al.*, 2019). *B. velezensis* BUU004 was cultured in a 250 mL flask containing 100 mL Trypticase Soy Broth (TSB; Becton, BD, Sparks, MD, USA) at 30°C for 24 h in an orbital shaker at 200 rpm (Nimrat *et al.*, 2008).

Food spoilage and food-borne pathogens used for evaluation of antibacterial activity included Gram-positive bacteria: *B. cereus* TISTR 687, *B. coagulans* ATCC 12245, *Listeria monocytogenes* ATCC 15313, *Micrococcus luteus* ATCC 9341, and *Staphylococcus aureus* ATCC 25923, and Gram-negative bacteria: *Escherichia coli* ATCC 25922, *E. coli* O157:H7 DMST 12743, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Typhimurium TISTR 292, and *Vibrio parahaemolyticus* ATCC 17802. All pathogenic bacteria were grown in Nutrient Broth (HiMedia Laboratories, Mumbai, India) at 37°C for 18 - 24 h.

Kinetics of antibacterial substance (ABS) production

A loopful of *B. velezensis* BUU004 was inoculated in 100 mL TSB and incubated at 200 rpm, 30°C in an incubator shaker. Cell growth and ABS production were measured by withdrawing cell suspension at desired time intervals during 48 h incubation (Ayed *et al.*, 2015). At each sampling, cell suspension was divided into two portions. For first portion, cell suspension was 10-fold diluted with 0.85% normal saline, and a volume (0.1 mL) of each dilution was plated onto Plate Count Agar (Difco, BD, Sparks, MD, USA). Following incubation at 35°C for 24 h, bacterial colonies were counted and calculated as colony forming unit per mL (CFU/mL). For second portion, cell suspension was centrifuged at 8,000 g, 4°C for 10 min (Centrifuge 5804 R, Eppendorf, Hamburg, Germany). Then, cell-free supernatant (CFS) was collected and filtered through a 0.45 μm pore-size filter (Sartorius, Goettingen, Germany). Antibacterial activity of CFS against *B. cereus* TISTR 687 used as indicator strain was evaluated using agar well diffusion method. Activity unit (AU) of ABS against *B. cereus* TISTR 687 was then calculated as the reciprocal of the highest dilution that gave a minimal visible inhibition zone against indicator bacteria and expressed as AU/mL (Avcı *et al.*, 2017).

Partial purification of ABS

The partially purified substance of *B. velezensis* BUU004 (PBV) was prepared from CFS collected at which the highest antibacterial activity of CFS was observed during 48 h incubation (18 h). After passing through a 0.45 μm filter, the CFS was precipitated with ammonium sulphate according to Avcı *et al.* (2017) with slight modification. Ammonium sulphate was added into CFS until reaching 80% saturation and kept overnight at 4°C with gentle stirring. Precipitated proteins were collected by centrifugation (10,000 g, 4°C, 30 min), dissolved in 50 mM sodium phosphate buffer (SPB) consisting of NaH_2PO_4 (7.742 g) and

$Na_2HPO_4 \cdot 7H_2O$ (2.914 g) in 1 L deionised water, pH 7.0) and then dialysed twice against the same buffer at 4°C for 24 h using a 1,000 kDa MWCO dialysis tubing (Spectrum Laboratory, New Brunswick, NJ, USA). The PBV was stored at -20°C until use.

Antibacterial spectrum of PBV

Antibacterial activity of PBV (800 AU/mL) and CFS was evaluated against pathogenic bacteria using agar well diffusion method (Abdhul *et al.*, 2015). Aliquots of 50 µL CFS or PBV were loaded into wells with 6 mm diameter made by a sterile cork borer on Müeller Hinton agar (Difco, BD, Sparks, MD, USA) plates seeded with each pathogenic strain (10^8 CFU/mL). Plates were incubated at 37°C for 24 h. Diameters of inhibition zone were then measured following incubation. The experiment was conducted in triplicates.

Detection of biochemical moieties in PBV

The presence of protein, carbohydrate and lipid molecules in PBV was evaluated using standard biochemical assays (Sawhney and Singh, 2001). Two drops of ninhydrin solution (2% w/v; Sigma-Aldrich, St. Louis, MO, USA) were added to 2 mL PBV, and the mixture was heated for 5 min in a boiling water bath. Blue-purple or yellow-orange appearance indicated the presence of amino acids and peptides. Arginine and ornithine (HiMedia) were used as positive control while distilled water was used as negative control.

In anthrone test, a mixture of 2 mL PBV and anthrone reagent (0.2% anthrone in conc. H_2SO_4; TCI Chemicals, Tokyo, Japan) was made. Bluish-green coloured formation was scored as positive, indicative of presence of a carbohydrate moiety. Glucose and starch solutions (5% w/v) were used as positive control with same condition.

Saponification test was achieved by adding equal volume of PBV, 98% ethanol and 2% NaOH solution (1 mL) in a test tube, and heating in boiling water for 15 min. The mixture was added with distilled water and then vigorously shaken. Formation of soap indicated the presence of lipid moiety. Olive oil and distilled water were used as positive and negative control, respectively.

Stability of PBV to enzyme, heat, and pH

The PBV (800 AU/mL) was tested for its sensitivity to enzymes, heat, and pH in triplicates according to Liu *et al.* (2015). To study the effect of proteolytic enzymes, PBV was treated with pepsin, trypsin, proteinase K, papain and chymotrypsin (Sigma-Aldrich) at a final concentration of 1 mg/

mL in SPB (50 mM, pH 7.0), except pepsin which was dissolved in 50 mM glycine HCl buffer (pH 2.0). Following incubation at 37°C for 2 h, PBV-enzyme mixtures were adjusted to pH 7.0 and heated at 70°C for 10 min to inactivate the enzymes. Then, antagonistic activity of enzyme treated PBV against *B. cereus* TISTR 687 was assessed as previously mentioned and expressed as residual activity. Untreated PBV in SPB, glycine HCl buffer, and buffers only were used as controls.

Thermal stability was studied by heating PBV dissolved in SPB (50 mM, pH 7.0) at 50, 60, 70, 80, 90, 92, 94, 96, 98, and 100°C for 30 min, and 121°C for 20 min. After cooling to room temperature, residual activity of PBV against *B. cereus* TISTR 687 was evaluated. Unheated PBV was used as control.

To evaluate the effect of pH, PBV was adjusted at various pH values from 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, by 5 N HCl or 1 N NaOH. Following incubation at 37°C for 2 h, each sample was neutralised to the pH 7.0 and filtered through a 0.45 µm filter. Residual activity of PBV against *B. cereus* TISTR 687 was determined using agar well diffusion. PBV in SPB (50 mM, pH 7.0) was used as control.

Molecular mass determination and identification of amino acid sequence

Molecular mass of a bacteriocin in PBV was preliminarily determined by Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE) with 5% stacking gel and 16% separating gel (Schägger, 2006) along with a protein standard marker (2.5 - 200 kDa; Mark12, Invitrogen, Carlsbad, CA, USA). Tricine SDS-PAGE was run for 30-60 min at 80 V, and then for 3-4 h at 120 V using a Mini-Protein 3 Cell (Bio-Rad, Hercules, CA, USA). Following electrophoresis, first half of the gel (protein gel) was stained with 0.025% Coomassie blue R-250 (ACROS Organics, Geel, Belgium) in 10% acetic acid, while the other half was washed three times with sterile distilled water for 30-40 min to remove SDS residue. Washed gel was overlaid with soft TSB agar (0.8% agar) containing *B. cereus* TISTR 687 cells (10^6 CFU/mL) and incubated at 37°C for 16-18 h (overlaid plate). The formation of inhibition zone on overlaid plate was observed and compared with the protein gel (Barboza-Corona *et al.*, 2007). The molecular mass of band with inhibition activity was estimated by comparing with molecular mass markers.

The single band located in protein gel with inhibitory activity according to the clear zone located in overlaid plate was cut from the gel for protein

identification. The protein in gel was digested with trypsin before subsequent LC-MS/MS analysis (Khochamit *et al.*, 2015). LC-MS/MS spectra were analysed using Compass Data Analysis v.4.0. Compound lists were exported as Mascot generic files (mgf) for further searching in the MASCOT MS/MS Ion Search program (www.matrixscience.com) and compared with protein sequences in National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.gov/protein).

In silico characterisation of toxin genes using nucleotide sequencing data

Whole genome sequencing of *B. velezensis* BUU004 was performed by Illumina HiSeq 4000 next-generation DNA sequencing platform (Illumina Inc., San Diego, CA, USA). The sequence reads were assembled using A5 assembly pipeline (https://chipster.csc.fi/manual/a5-miseq.html; Tritt *et al.*, 2012). The NCBI Prokaryotic Genomes Automatic Annotation Pipeline was used to perform the gene annotation (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/; Tatusova *et al.*, 2016). The annotated genome assembly is available in the NCBI as Whole Genome Shotgun project under the GenBank accession number SJCZ01000000 and BioProject ID in NCBI is PRJNA508842. In a next step, the presence of toxin genes (diarrhoeal enterotoxin and emetic toxins) involved in gastrointestinal infections reported in *B. cereus* and other *Bacillus* species were determined according to FDA (2012) and EFSA (2014) guidance. Diarrhoeal enterotoxin genes, i.e. cytotoxin K (*cytK*), hemolysin (*hlyII*), hemolytic enterotoxin HBL (*hblABCD*), and non-hemolytic enterotoxin NHE (*nheABC*) were identified using encoding genes via a comparison to the virulence factor database (VFDB; http://www.mgc.ac.cn/VFs/; Liu *et al.*, 2018) using automatic and comprehensive platform (VFanalyzer) for accurate bacterial virulence factor identification. Moreover, the presence of emetic toxin genes (*cesABCD*) was analysed using BTyper version 2.3.0 (https://github.com/lmc297/BTyper; Carroll *et al.*, 2017). The presence of toxin genes was compared to those of the reference strains: *B. cereus* ATCC 14579, *B. licheniformis* ATCC 14580, *B. subtilis* subsp. *subtilis* 168, and *B. mycoides* DSM 2048.

Cytotoxicity assay

Cytotoxic impact of PBV and nisin (Sigma-Aldrich) on the viability of human kidney cell line (HK-2) was evaluated using a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Pujalté *et al.*, 2011).

Briefly, HK-2 cells obtained from the American Type Culture Collection (ATCC) were grown in a 96-well microplate (Costar, Cambridge, MA, USA) containing Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific). Following incubation at 37°C, for 24 h in a CO_2 incubator, culture medium was discarded. Afterwards, a small volume (100 μL) of DMEM containing various concentrations of PBV (312.5-20,000 μg/mL) or nisin (45-3,000 μg/mL) was inoculated in triplicate wells of 96-well microplate containing monolayer of confluent HK-2 cells. Plates were incubated at 37°C for 24 h. Then, culture medium containing tested agents was removed and serum-free DMEM plus 0.5 mg/mL MTT was added into each well. After 3 h incubation, DMSO (100 μL) was added into the wells to dissolve formazan crystals. Absorbance was measured at 570 nm using a multimode plate reader (EnVision, Perkin-Elmer, Waltham, MA, USA). HK-2 cell treated with DMEM served as negative control. Percent viability of HK-2 cell treated with tested agents at different concentrations was calculated using the following formula.

$$\% \text{ Viability} = \frac{(\text{A570 of treated cells } - \text{ A570 of blank cells})}{(\text{A570 of controlled cells } - \text{ A570 of blank cells})} \times 100$$

The IC_{50} values (the concentration of tested agents required to kill 50% of the cells) were then calculated using the non-linear regression analysis of the GraphPad Prism version 7.04 for Window (GraphPad Software, San Diego, CA, USA).

Statistical analysis

All experiments were performed in triplicates. Data were presented as mean ± standard deviation. The IC_{50} values for MTT cytotoxicity assay were determined using non-linear regression analysis with variable slope sigmoidal curve fitting model provided by the GraphPad Prism 7.04 software.

Results and discussion

Kinetics of ABS production and cell growth

The relationship between cell growth and inhibitory activity of ABS produced by *B. velezensis* BUU004 is shown in Figure 1. Inhibitory activity against *B. cereus* TISTR 687 was growth-associated and began in the early stationary growth phase at 8 h post-cultivation (6.8 ± 0.3 mm of inhibition zone).
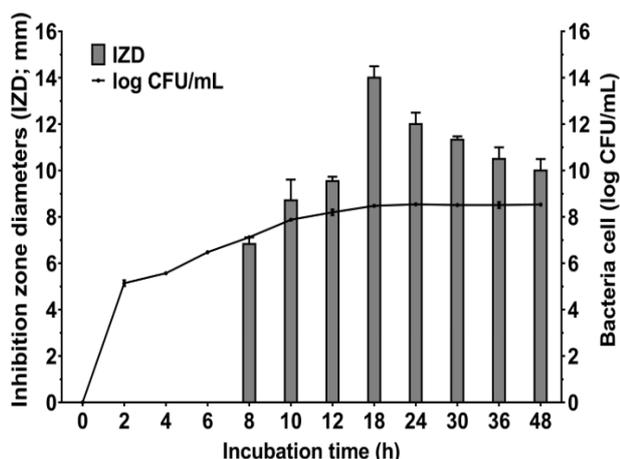
Figure 1. Kinetics of cell growth and inhibitory activity of antibacterial substances produced by *Bacillus velezensis* BUU004 against *B. cereus* TISTR 687. Data are means ± S.D.

The maximum activity at 14.0 ± 0.5 mm of inhibition zone was recorded at the middle of stationary growth phase at 18 h of cultivation. Afterwards, the inhibitory activity of ABS progressively declined. Antibacterial substance produced by bacteria is one of the secondary metabolites synthesised during exponential or stationary growth phases (Sansinenea and Ortiz, 2011). Growth-associated relationship in ABS production was reported in other *Bacillus* species. Antibacterial secondary metabolites were produced by *B. amyloliquefaciens* N2-4 and N3-8, a closely related species within *B. subtilis* group, at the early stationary growth phase and reached a maximum level at mid-stationary growth phase (Boottanun *et al.*, 2017). Kleerebezem (2004) described that ABS were produced and rapidly increased to maximal level at the stationary growth phase to ensure that their concentrations were enough to control the competitor cells in competitive environments.

Antibacterial activity

The broad antibacterial spectrum of PBV against important food spoilage and foodborne bacteria is presented in Table 1. This partially purified substance strongly inhibited all the tested Gram-positive bacteria (*B. cereus* TISTR 687, *B. coagulans* ATCC 12245, *L. monocytogenes* ATCC 15313, *M. luteus* ATCC 9341, and *S. aureus* ATCC 25923) with inhibition zone ranging from 10.7 ± 1.15 to 17.8 ± 0.29 mm. Three tested Gram-negative bacteria strains (*E. coli* ATCC 25922, *E. coli* O157:H7 DMST 12743, and *Salmonella* Typhimurium TISTR 292) were obviously inhibited by PBV with inhibition zone between 8.2 ± 0.29 and 12.5 ± 0.50 mm. PBV exhibited weak inhibitory effect on *K. pneumoniae* ATCC 13883. However, inhibition zones were observed in neither *P. aeruginosa* ATCC

27853 nor *V. parahaemolyticus* ATCC 17802 after PBV was applied. These results suggested that PBV had a wide inhibitory spectrum against food spoilage and pathogenic bacteria. Similarly, bacteriocins, namely amylolysin and amylocyclicin produced by *B. velezensis* LS69 strain, were found to inhibit both Gram-positive and Gram-negative pathogenic bacteria, e.g. *B. cereus*, *B. thuringiensis*, *S. aureus*, *L. monocytogenes*, *Clostridium perfringens*, *E. coli* DH5α, and *P. putida* (Liu *et al.*, 2017).

Table 1. Antimicrobial spectrum of partially purified substance from *Bacillus velezensis* BUU004.

Indicator strain	Source[a]	Inhibition zone (mm ± S.D)*
Gram-positive bacteria		
Bacillus cereus	TISTR 687	14.2 ± 0.76
B. coagulans	ATCC 12245	15.0 ± 0.50
Listeria monocytogenes	ATCC 15313	17.5 ± 0.50
Micrococcus luteus	ATCC 9341	17.8 ± 0.29
Staphylococcus aureus	ATCC 25923	10.7 ± 1.15
Gram-negative bacteria		
Escherichia coli	ATCC 25922	11.3 ± 0.29
E. coli O157:H7	DMST 12743	8.2 ± 0.29
Klebsiella pneumoniae	ATCC 13883	6.8 ± 0.29
Pseudomonas aeruginosa	ATCC 27853	-
Salmonella Typhimurium	TISTR 292	12.5 ± 0.50
Vibrio parahaemolyticus	ATCC 17802	-

[a]TISTR: Thailand Institute of Scientific and Technological Research Culture Collection; ATCC: American Type Culture Collection; DMST: Department of Medical Sciences, Thailand Culture Collection. *Diameter of the inhibition zone (mm) around the well (the diameter of each well was 6.0 mm).

Biochemical moieties in ABS from B. velezensis BUU004

The analyses of biochemical moieties revealed that PBV prepared from *B. velezensis* BUU004 had peptide and/or amino acid residues as evidenced by the formation of yellow-orange solution (Friedman, 2004). In addition, carbohydrate and lipid moieties were absent in the PBV owing to negative reactions produced. These results confirm the proteinaceous nature without sugar and lipid moieties of a bacteriocin in PBV produced by *B. velezensis* BUU004.

Stability of PBV to enzymes, heat, and pH

The inhibitory activity of PBV against *B. cereus* TISTR 687 used as indicator strain was stopped following the exposure to pepsin, proteinase K and trypsin. Meanwhile, PBV was partially inactivated when treated with papain and chymotrypsin, showing $67 \pm 6.0\%$ and $74 \pm 3.9\%$ of residual activity (Figure

2A). Degradation of PBV by proteolytic enzymes revealed that antibacterial activity of PBV was related to active antibacterial peptides. PBV was very sensitive to proteolytic enzyme treatments, thereby demonstrating its proteinaceous nature.

Antibacterial activity of PBV against *B. cereus* TISTR 687 remained highly stable after 30 min incubation at temperature between 50 and 70°C. A slight decrease in inhibitory activity was noticeable at 80 and 90°C with residual activity of $86 \pm 2.6\%$ and $75 \pm 2.4\%$, respectively. The residual activity of PBV remained $49 \pm 2.4\%$ at 96°C and vanished when heating at 98°C for 30 min and autoclaving at 121°C for 20 min (Figure 2B). Bacteriocin-like substances from *B. amyloliquefaciens* strain ELI149 and An6 were also reported to have a heat-stable property at temperatures up to 100°C (Ayed *et al.*, 2015; Salazar *et al.*, 2017).
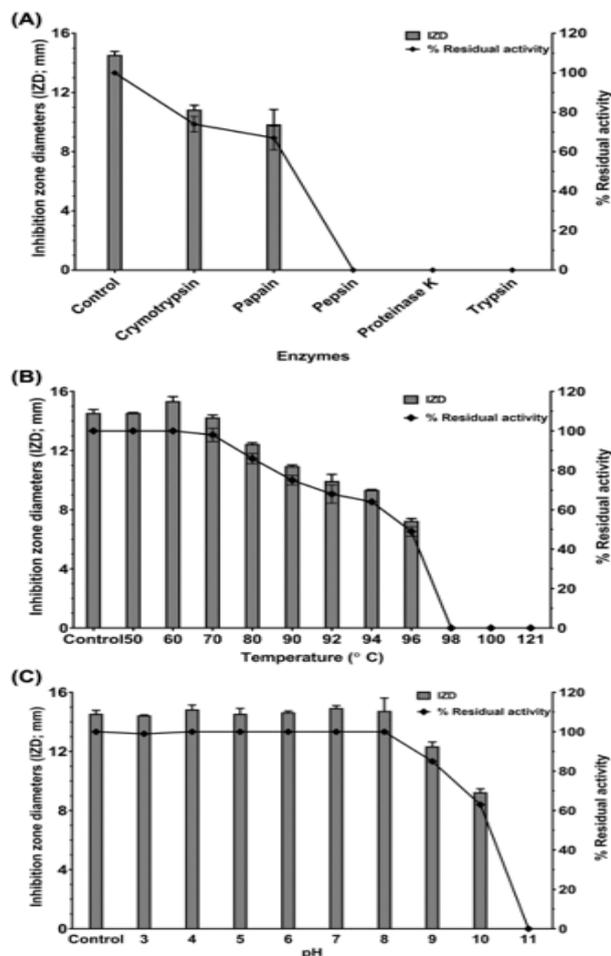

Figure 2. Effect of enzymes (A), temperatures (B) and pH (C) on antibacterial activity of partially purified substance produced by *Bacillus velezensis* BUU004 (PBV) against *B. cereus* TISTR 687. The residual activity (%) was calculated as follows: % residual activity = $100\% \times (X/C)$, where C = an antibacterial activity of the control and X = an antibacterial activity of sample. Data are means ± S.D.

The residual activity of PBV at different pH values is illustrated in Figure 2C. Antibacterial activity of PBV was relatively stable at a wide pH range (3.0-9.0), but decreased at pH 10.0 and vanished completely at pH 11.0. The maximum activity was found at pH 7.0. These results were consistent with several reports focused on bacteriocin production by *B. amyloliquefaciens, i.e.* subtilosin A and B, and BLIS production by *B. amyloliquefaciens* ELI149 showing pH stability in a wide range from 2.0 - 10.0 (Liu *et al.*, 2012; Salazar *et al.*, 2017).

Due to its proteinaceous nature without lipid and sugar moieties in the structure, ABS produced by *B. velezensis* BUU004 was classified as bacteriocin with thermoresistance up to 96°C and stability between pH 3.0 and 9.0.
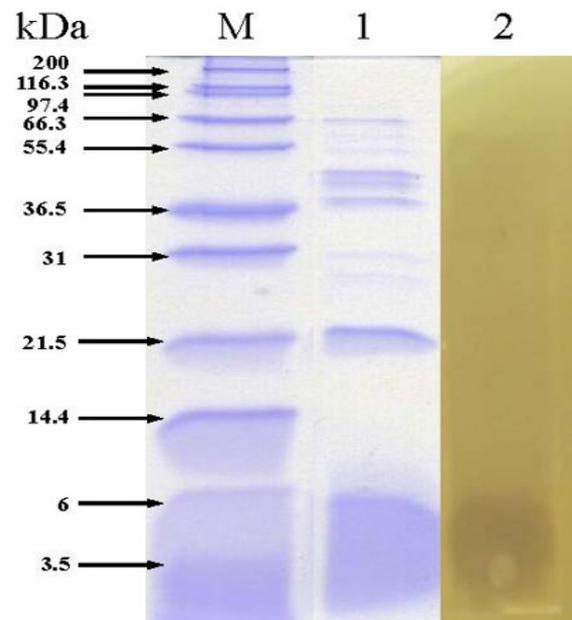

Figure 3. Tricine–SDS-PAGE analysis and direct detection of antibacterial activity. lane M: molecular weight marker; lane 1: partially purified substance from *Bacillus velezensis* BUU004 (PBV); and lane 2: inhibition zone against *B. cereus* TISTR 687 of a single band in overlaid plate with soft Trypticase Soy Agar.

Molecular mass and protein identification

The molecular weight of a bacteriocin in PBV was assessed by Tricine SDS-PAGE analysis. As depicted in Figure 3, a single peptide band with halo zone represented inhibitory activity against indicator strain, *B. cereus* TISTR 687. The band had a molecular mass of approximately 5.75 kDa. Amino acid sequence of the antibacterial peptides was determined by LC-MS/MS analysis and compared to the protein database from NCBI. Amino acid sequence of bacteriocin in PBV showed 43% protein coverage similar to that of a portion of hypothetical protein belonging to

Table 2. Presence and absence of putative virulence factors of *Bacillus* toxins in *Bacillus velezensis* BUU004 genome.

Virulence factors of *Bacillus* toxins	Related genes	*B. velezensis* BUU004	*B. cereus* ATCC 14579	*B. licheniformis* ATCC 14580	*B. subtilis* subsp. *subtilis* 168	*B. mycoides* DSM 2048
Cytotoxin K	*cytK*	-	+	-	-	+
Hemolysin II	*hlyII*	-	+	-	-	+
Hemolytic enterotoxin HBL	*hblA*	-	+	-	-	+
	hblB	-	+	-	-	+
	hblC	-	+	-	-	+
	hblD	-	+	-	-	+
Non-hemolytic enterotoxin NHE	*nheA*	-	+	-	-	+
	nheB	-	+	-	-	+
	nheC	-	+	-	-	+
Emetic toxin	*cesA*	-	-	-	-	-
	cesB	-	-	-	-	-
	cesC	-	-	-	-	+
	cesD	-	-	-	-	-

+: present; -: absent

B. subtilis group (WP_072589202.1 in NCBIprot) deposited in the database. This suggests that our substance is possibly a new bacteriocin and has not been previously reported its structure and function in the available database. Several strains of *B. velezensis* were found to be capable of biosynthesising a variety of bacteriocins: a 6.38 kDa circular amylocyclicin and a 1.33 kDa plantazolicin by *B. velezensis* FZB42, formerly known as *B. amyloliquefaciens* (Scholz *et al.*, 2011; 2014), ericin S by *B. velezensis* RC 218 (Palazzini *et al.*, 2016) and type II lantipeptides by *B. velezensis* 9912D (Pan *et al.*, 2017).

Presence of toxin genes

B. velezensis BUU004 carried none of the cytotoxin K (*cytK*), hemolysin (*hlyII*), hemolytic enterotoxin HBL (*hblABCD*), non-hemolytic enterotoxin NHE (*nheABC*) and cereulide (*cesABCD*) genes (Table 2). In fact, the presence of toxin genes is one of the most important traits for biosafety evaluation of *Bacillus* species intended to be used in human and animal feed productions since it is a major causative agent of gastrointestinal diseases and enterotoxigenic potential of bacteria (FDA, 2012). Enterotoxins, i.e. hemolytic enterotoxin HBL, non-hemolytic enterotoxin NHE, cytotoxin K, and cereulide have been frequently found in *Bacillus* species such as *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. anthracis*, and are considered as virulence factors because their ability to cause diarrhoeal and emetic syndromes (Stenfors Arnesen *et al.*, 2008; Jeßberger *et al.*, 2015). This result was in agreement with Yi *et al.* (2018) who also reported the absence of enterotoxin genes in *B. velezensis* JW strain, an antimicrobial peptide producer.

Cytotoxicity

Cytotoxicity of PBV prepared from *B. velezensis* BUU004 was tested *in vitro* using HK-2 cells. IC_{50} values of PBV and nisin to HK-2 cells were 927.4 and > 3,000 μg/mL, respectively. Bacteriocins and BLIS from *Bacillus* strains have been reported to have variations in cytotoxicity on mammalian cell lines. According to Vaucher *et al.* (2010), IC_{50} of purified bacteriocin obtained from *B. licheniformis* P40 on Vero cells (Vero monkey kidney cells) was 0.30 μg/mL. Also, Abdhul *et al.* (2015) reported that the bacteriocin from *B. coagulans* BDU3 had low cytotoxicity at 2.0 μg/mL to human embryonic kidney (HEK 293) cells. PBV prepared from our isolate seemed to have lower toxicity to kidney cell lines in comparison with previously reported values. The discrepancy among cytotoxicity studies of bacteriocins and BLIS was possibly related to differences in membrane composition, cell surface hydrophobicity, and metabolic activity of tested cell lines, varying exposure times, and cytotoxicity assay employed (Vaucher *et al.*, 2010). Moreover, cytotoxicity observed in the present work was not associated with the harmful toxins frequently found in *B. cereus* and other *Bacillus* strains because of absence of the enterotoxin genes as aforementioned. The actual mechanism involved in cytotoxicity of our PBV remains unknown and merits to be further investigated.

The absence of the virulence genes involved in the gastrointestinal disorders and low cytotoxicity pointed out that *B. velezensis* BUU004 could be granted the QPS status following the recommended criteria prescribed by EFSA (2014). It is therefore

reasonable to highlight that *B. velezensis* BUU004 possesses a basic biosafety feature, has no harmful risk to human health, and can be safely used in human and animal nutrition. In the future study, PBV should be evaluated for toxicological testing such as short-term and sub-chronic toxicity, and genotoxicological safety evaluation to further ensure its biosafety before being applied in human and animal food products.

Conclusion

The maximum inhibitory activity of PBV was produced at the middle of stationary growth phase (18 h post-incubation). Bacteriocin in PBV was digested by proteolytic enzymes, thus revealing its proteinaceous nature. The bacteriocin had low molecular weight (ca. 5.75 kDa), showed stability over a broad range of pH (3.0 - 9.0) and temperatures (50 - 96°C), and inhibited several species of Gram-positive and Gram-negative foodborne pathogenic bacteria. Based on biosafety assessment, PBV displayed low cytotoxic effect on HK-2 cells and did not represent a risk to human health due to absence of the enterotoxin genes in its genome. Thus, bacteriocin in PBV has a great potential as a natural food biopreservative and as an alternative to current chemical counterpart to improve bacteriological quality of food products.

Acknowledgement

References

Abdhul, K., Ganesh, M., Shanmughapriya, S., Vanithamani, S., Kanagavel, M., Anbarasu, K. and Natarajaseenivasan, K. 2015. Bacteriocinogenic potential of a probiotic strain *Bacillus coagulans* [BDU3] from Ngari. International Journal of Biological Macromolecules 79: 800-806.

Abriouel, H., Franz, C. M., Ben Omar, N. and Gálvez, A. 2011. Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiology Reviews 35(1): 201-232.

Avcı, A., Çağrı-Mehmetoğlu, A. and Arslan, D. 2017. Production of antimicrobial substances by a novel *Bacillus* strain inhibiting *Salmonella* Typhimurium. LWT 80: 265-270.

Ayed, H. B., Maalej, H., Hmidet, N. and Nasri, M. 2015. Isolation and biochemical characterisation of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* An6. Journal of Global Antimicrobial Resistance 3(4): 255-261.

Barboza-Corona, J. E., Vázquez-Acosta, H., Bideshi, D. K. and Salcedo-Hernández, R. 2007. Bacteriocin-like inhibitor substances produced by Mexican strains of *Bacillus thuringiensis*. Archives of Microbiology 187(2): 117-126.

Boottanun, P., Potisap, C., Hurdle, J. G. and Sermswan, R. W. 2017. Secondary metabolites from *Bacillus amyloliquefaciens* isolated from soil can kill *Burkholderia pseudomallei*. AMB Express 7: article ID 16.

Butkhot, N., Soodsawaeng, P., Samutsan, S., Chotmongcol, K., Vuthiphandchai, V., and Nimrat, S. 2019. New perspectives for surveying and improving Thai dried seafood qualities using antimicrobials produced by *Bacillus velezensis* BUU004 against foodborne pathogens. ScienceAsia 45(2):116-26.

Carroll, L. M., Kovac, J., Miller, R. A. and Wiedmann, M. 2017. Rapid, high-throughput identification of anthrax-causing and emetic *Bacillus cereus* group genome assemblies via BTyper, a computational tool for virulence-based classification of *Bacillus cereus* group isolates by using nucleotide sequencing data. Applied and Environmental Microbiology 83(17): article ID e01096-17.

European Food Safety Authority (EFSA). 2014. Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. EFSA Journal 12(5): article ID 3665.

Fan, B., Blom, J., Klenk, H. P. and Borriss, R. 2017. *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* form an "operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. Frontiers in Microbiology 8: article ID 22.

Food and Drug Administration (FDA). 2012. Bad bug book. Foodborne pathogenic microorganisms and natural toxins handbook. Retrieved on March 18, 2019 from FDA website: https://www.fda.gov/media/83271/download

Friedman, M. 2004. Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. Journal of Agricultural and Food Chemistry 52(3): 385-406.

Harwood, C. R., Mouillon, J. M., Pohl, S. and Arnau, J. 2018. Secondary metabolite production and the safety of industrially important members of the *Bacillus subtilis* group. FEMS Microbiology Reviews 42(6): 721-738.

Jeßberger, N., Krey, V. M., Rademacher, C., Böhm, M. E., Mohr, A. K., Ehling-Schulz, M., ... and Märtlbauer, E. 2015. From genome to toxicity: a combinatory approach highlights the complexity of enterotoxin production in *Bacillus cereus*. Frontiers in Microbiology 6: article ID 560.

Johnson, E. M., Jung, Y. G., Jin, Y. Y., Jayabalan, R., Yang, S. H. and Suh, J. W. 2018. Bacteriocins as food preservatives: challenges and emerging horizons. Critical Reviews in Food Science and Nutrition 58(16): 2743-2767.

Khochamit, N., Siripornadulsil, S., Sukon, P. and Siripornadulsil, W. 2015. Antibacterial activity and genotypic–phenotypic characteristics of bacteriocin-producing *Bacillus subtilis* KKU213: potential as a probiotic strain. Microbiological Research 170: 36-50.

Kleerebezem, M. 2004. Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis. Peptides 25(9): 1405-1414.

Liu, B., Zheng, D., Jin, Q., Chen, L. and Yang, J. 2018. VFDB 2019: a comparative pathogenomic platform with an interactive web interface. Nucleic Acids Research 47(D1): D687-D692.

Liu, G., Kong, Y., Fan, Y., Geng, C., Peng, D. and Sun, M. 2017. Whole-genome sequencing of *Bacillus velezensis* LS69, a strain with a broad inhibitory spectrum against pathogenic bacteria. Journal of Biotechnology 249: 20-24.

Liu, Q., Gao, G., Xu, H. and Qiao, M. 2012. Identification of the bacteriocin subtilosin A and loss of *purL* results in its high-level production in *Bacillus amyloliquefaciens*. Research in Microbiology 163(6-7): 470-478.

Liu, X., Lee, J. Y., Jeong, S. J., Cho, K. M., Kim, G. M., Shin, J. H., ... and Kim, J. H. 2015. Properties of a bacteriocin produced by *Bacillus subtilis* EMD4 isolated from Ganjang (soy sauce). Journal of Microbiology and Biotechnology 25(9): 1493-1501.

Nimrat, S., Suksawat, S., Maleeweach, P. and Vuthiphandchai, V. 2008. Effect of different shrimp pond bottom soil treatments on the change of physical characteristics and pathogenic bacteria in pond bottom soil. Aquaculture 285(1-4): 123-129.

Palazzini, J. M., Dunlap, C. A., Bowman, M. J. and Chulze, S. N. 2016. *Bacillus velezensis* RC 218 as a biocontrol agent to reduce *Fusarium* head blight and deoxynivalenol accumulation: genome sequencing and secondary metabolite cluster profiles. Microbiological Research 192: 30-36.

Pan, H.Q., Li, Q.L. and Hu, J.C. 2017. The complete genome sequence of *Bacillus velezensis* 9912D reveals its biocontrol mechanism as a novel commercial biological fungicide agent. Journal of Biotechnology 247: 25-28.

Pujalté, I., Passagne, I., Brouillaud, B., Tréguer, M., Durand, E., Ohayon-Courtès, C. and L'Azou, B. 2011. Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells. Particle and Fibre Toxicology 8: article ID 10.

Salazar, F., Ortiz, A. and Sansinenea, E. 2017. Characterization of two novel bacteriocin-like substances produced by *Bacillus amyloliquefaciens* ELI149 with broad spectrum antimicrobial activity. Journal of Global Antimicrobial Resistance 11: 177-182.

Sansinenea, E. and Ortiz, A. 2011. Secondary metabolites of soil *Bacillus* spp. Biotechnology Letters 33(8): 1523-1538.

Sawhney, S. K. and Singh, R. 2001. Introductory practical biochemistry. India: Narosa Publishing House.

Schägger, H. 2006. Tricine-SDS-PAGE. Nature Protocols 1(1): 16-22.

Scholz, R., Molohon, K. J., Nachtigall, J., Vater, J., Markley, A. L., Süssmuth, R. D., ... and Borriss, R. 2011. Plantazolicin, a novel microcin B17/streptolysin S-like natural product from *Bacillus amyloliquefaciens* FZB42. Journal of Bacteriology 193(1): 215-224.

Scholz, R., Vater, J., Budiharjo, A., Wang, Z., He, Y., Dietel, K., ... and Borriss, R. 2014. Amylocyclicin, a novel circular bacteriocin produced by *Bacillus amyloliquefaciens* FZB42. Journal of Bacteriology 196(10): 1842-1852.

Settanni, L. and Corsetti, A. 2008. Application of bacteriocins in vegetable food biopreservation. International Journal of Food Microbiology 121(2): 123-138.

Sewalt, V., Shanahan, D., Gregg, L., La Marta, J. and Carrillo, R. 2016. The Generally Recognized as Safe (GRAS) process for industrial microbial enzymes. Industrial Biotechnology 12(5): 295-302.

Stenfors Arnesen, L. P., Fagerlund, A. and Granum, P. E. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiology Reviews 32(4): 579-606.

Tagg, J. R., Dajani, A. S. and Wannamaker, L. W. 1976. Bacteriocins of gram-positive bacteria. Bacteriological Reviews 40(3): 722-756.

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., ... and Ostell, J. 2016. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Research 44(14): 6614-6624.

Tritt, A., Eisen, J. A., Facciotti, M. T. and Darling, A. E. 2012. An integrated pipeline for de novo assembly of microbial genomes. PLoS ONE 7(9): article ID e42304.

Vaucher, R. A., Teixeira, M. L. and Brandelli, A. 2010. Investigation of the cytotoxicity of antimicrobial peptide P40 on eukaryotic cells. Current Microbiology 60(1): 1-5.

Yang, S.C., Lin, C.H., Sung, C. T. and Fang, J.Y. 2014. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Frontiers in Microbiology 5: article ID 241.

Yi, Y., Zhang, Z., Zhao, F., Liu, H., Yu, L., Zha, J. and Wang, G. 2018. Probiotic potential of *Bacillus velezensis* JW: antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*. Fish and Shellfish Immunology 78: 322-330.