International Journal of Biological Macromolecules xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Bacteriocinogenic potential of a probiotic strain *Bacillus coagulans* [BDU3] from Ngari

Q1 Kaja Abdhul[a,b], Mohan Ganesh[a], Santhanam Shanmughapriya[a,1], Shanmugam Vanithamani[a], Murugesan Kanagavel[a], Kumarasamy Anbarasu[c], Kalimuthusamy Natarajaseenivasan[a,*]

[a] *Medical Microbiology Laboratory, Department of Microbiology, Centre for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli 620 024, India*
[b] *Department of Biotechnology, Nandha Arts and Science College, Bharathiar University, Erode 638009, India*
[c] *Microbial Technology Laboratory, Department of Marine Biotechnology, Centre for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli 620024, India*

ARTICLE INFO

Article history:
Received 28 January 2015
Received in revised form 29 May 2015
Accepted 2 June 2015
Available online xxx

Keywords:
Probiotics
Bacteriocin
Bacillus coagulans
Ngari
FTIR
MALDI-TOF

ABSTRACT

Bacteriocin producing strain BDU3 was isolated from a traditional fermented fish of Manipur Ngari. The strain BDU3 was identified as *Bacillus coagulans* by phenotypic and genotypic characterization. The BDU3 produced novel bacteriocin, which showed an antimicrobial spectrum toward a wide spectrum of food borne, and closely related pathogens with a MIC that ranged between 0.5 and 2.5 μg/mL. The isolate was able to tolerate pH as low as 2.0 and up to 0.2% bile salt concentration. Three step purification was employed to increase the specific activity of the antimicrobial compound. The fractions were further chromatographed by R_p-HPLC C-18 column and the purified bacteriocin had a specific activity of ~8500 AU/mg. However, the potency of bacteriocin was susceptible to digestion with Proteinase K, Pepsin, SDS, EDTA and Urea. Molecular mass of purified bacteriocin was found to be 1.4 kDa using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). The functional group was revealed by FTIR analysis. The cytotoxicity assay (MTT) using purified bacteriocin showed 2 times lower EC50 values compared to SDS. This is the smaller bacteriocin ever reported before from *B. coagulans* with greater antimicrobial potency with lower cytotoxicity. This bacteriocin raises the possibilities to be used as a biopreservative in food industries.

1. Introduction

Q2 Infectious diseases along with multidrug resistance are the major public health problem in developing countries with increased mortality and morbidity [1]. Apart from the threat of multidrug resistance, several studies have confirmed that the continuous use of antibiotics can damage human commensal micro flora [2]. The antibiotic pipeline has almost exhausted, and an alternative and effective research focus is necessary to combat these pathogens with no effect on normal flora. In this regard, the use of probiotics and their natural metabolic compounds can be a substitute in various food and pharmaceutical industries.

Bacteriocins are an abundant and diverse group of ribosomally synthesized antimicrobial peptides produced by bacteria and Archaea [3]. The bacteriocins were first identified as heat-labile product called as colicin present in cultures of *E. coli* V and toxic to *E. coli* S [4]. Recently, bacteriocins from lactic acid bacteria (LAB) have received much attention as a natural food preservative and a potential therapeutic antibiotic [5,6]. Application of bacteriocins for the control of some pathogens and food spoilage organisms has been approved in a number of countries [7–9]. Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. lactis from dairy products, has been extensively investigated and approved for use as a food preservative for more than 40 years in over 50 countries [10,11].

Production of bacteriocins or bacteriocin-like inhibitory substances (BLIS) by several species within the *Bacillus* genus has been reported by several research groups. Polyfermenticin SCD is a heat-labile, proteinase K-sensitive bacteriocin produced by *Bacillus polyfermenticus* SCD [12]. Subtilin is a lantibiotic-type bacteriocin produced by a strain of *Bacillus subtilis* with inhibitory activity

* Corresponding author. Tel.: +91 431 2407082; fax: +91 431 2407045.
E-mail address: natarajaseenivasan@rediffmail.com (K. Natarajaseenivasan).
[1] Present address: Center for Translational Medicine, School of Medicine, Temple University, Philadelphia, PA 19140, USA.

http://dx.doi.org/10.1016/j.ijbiomac.2015.06.005
0141-8130/

against a wide range of Gram-positive bacteria [13]. Subtilosin A is a ribosomally synthesized and post-translationally modified antimicrobial peptide produced by *B. subtilis* and active against a variety of Gram-positive bacteria including the foodborne pathogen *L. monocytogenes* [14]. Riazi et al. (2009) [15] reported lactosporin production from *Bacillus coagulans* isolated from a probiotic dietary supplement, Lactospore® probiotic.

With this view point and as a continuation of our previous attempt of isolating an efficient antioxidant probiotic *Enterococcus faecium* BDU7 [16] in the present study, we opted to study the bacteriocinogenic potential of a probiotic *B. coagulans* from "Ngari" a fermented fish product which has been traditionally consumed in Manipur.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacillus coagulans sp., BDU3 was isolated from Ngari (a fermented fish product) as described previously [16]. It was selected among several other co-isolated Gram-positive and catalase positive bacteria for its antibacterial potency. The isolate BDU3 was grown and maintained at 37 °C in nutrient broth or agar unless otherwise stated. *Salmonella enterica* serovar Typhi MTCC 733 was obtained from the Institute of Microbial Technology, Chandigarh, maintained in Growth Medium 3 and was used as indicator strain for antagonistic tests.

2.2. Characterization of bacteriocinogenic-probiotic isolate BDU3

The isolate was identified biochemically [17]. The ability of the isolate to grow at different temperature (range 4–70 °C), NaCl concentration (1–5%), and pH (2–8) was analyzed [18]. The probiotic potential of the isolate was verified by its acid and bile tolerance as described previously [19]. The 16S rRNA based genotype identification of the isolate was carried out as described earlier [16]. The 16S rRNA sequences thus obtained was submitted in GenBank with the accession number JX847608.

2.3. Screening for antimicrobial potency of B. coagulans BDU3

The double-agar layer method was used; *B. coagulans* BDU3 was streaked onto the surface of MRS agar and after incubation at 30 °C overnight, 10 mL of soft Trypticase soy agar (TSA) seeded with an overnight culture of indicator strain (*S. typhi* MTCC 733), was overlayed. After agar solidification, the plates were incubated for 18 h at 37 °C and examined for the presence of inhibition zones. Inhibition halos indicate a putative bioactive compound production.

Further, the antimicrobial potency of the culture free supernatant was verified. Briefly, 10 mL of overnight culture of BDU3 was inoculated into 500 mL MRS broth. The culture was centrifuged at 15,000 rpm at 4 °C, and the supernatant was filter sterilized. The cell free supernatant (CFS) thus obtained was screened for bioactive potential by agar well diffusion method [20]. A panel of microbes was used to test the antimicrobial potency of CFS. An overnight culture of the test strains was inoculated on MRS soft agar. 100 μL CFS were poured into the wells and the plates were incubated at 37 °C. After 24 h of incubation, the diameter of the zone of inhibition was measured and scored.

2.4. Protease susceptibility of the antimicrobial compound from B. coagulans BDU3

To confirm if the bioactives produced by isolate BDU3 was proteinaceous, the susceptibility of the compound to proteolytic degradation was tested [21]. A loopful of an overnight culture was spotted on TSA plates. After incubation at 37 °C for 16 h, 20 μL of a 20 μg/mL enzyme aqueous solution (Protease type XIV from *Streptomyces griseus*, Proteinase K from *Tritirachium album* and a-Chymotrypsin from bovine pancreas) (Sigma–Aldrich, USA) were applied in 2 mm diameter wells cut on the agar surface. Sterile MQ water was used as a negative control. After incubation at 30 °C for 2 h, the plates were overlaid with 10 mL of soft TSA seeded with *S. typhi* MTCC 733. The plates were incubated at 37 °C for 24 h. The absence of inhibition halo next to the well containing proteolytic enzyme indicates the proteinaceous nature of the antimicrobial compound.

Further the effect of enzymes (20 μg/mL) and detergents (1% v/v) on the bioactive potential of the CFS was studied. The crude CFS was pre-treated for 1 h with various enzymes and detergents (as indicated in Table 2) and tested for its antimicrobial potency. Effects of pH were determined by adjusting the pH of CFS with diluted HCl/NaOH. Samples were incubated for 2 h at various pH at 37 °C, readjusted to pH 6.5 before antimicrobial assay. The effect of various temperatures was determined by incubating at CFS 30, 45 and 60 °C under regulated pH 6.0. The antimicrobial potency of CFS was expressed as arbitrary units (AU). AU was analyzed by spot-on-lawn method [22] using *S. typhi* MTCC 733 as an indicator. After overnight incubation at 37 °C, the titer was defined as the reciprocal of the highest dilution that resulted in the inhibitor lawn. AU of antibacterial activity per milliliter was calculated by $2^n \times 1000\ \mu L/10\ \mu L$.

2.5. Growth and bacteriocin production kinetics

The optimal growth and bacteriocin production kinetics was determined. MRS broth was inoculated with 1% overnight culture of *B. coagulans* BDU3. The growth was measured at regular intervals of 2 h by measuring the $OD_{600\,nm}$ (BioPhotomer Plus, Eppendorf, Germany). The inhibitory activity at each time interval was assessed using agar well-diffusion test.

2.6. Production and purification of the bacteriocins

Ten milliliters of an overnight culture of *B. coagulans* BDU3 was inoculated into 500 mL of MRS broth (Hi media), incubated at 37 °C for 24 h. The culture was centrifuged at 15,000 rpm at 4 °C (Sigma, Germany), pH of the supernatant was adjusted to 7 with 5 M NaOH and filter sterilized (0.22 μm; crude bacteriocin). The sterile culture supernatant was treated with 60% ammonium sulphate for protein precipitation and the mixture stirred gently at 4 °C overnight. The precipitated protein was pelleted by centrifugation at 10,000 rpm for 10 min (partially purified bacteriocin). The partially purified bacteriocin was resuspended in 0.05 M phosphate buffer and dialyzed against deionized water at 4 °C overnight (purified bacteriocins). These purified bacteriocins were applied to C_{18} reverse phase column (10 × 250 mm). Mobile phase A (Water/TFA, 90:10 v/v) and mobile phase B (acetonitrile/water/TFA, 80:19.95:0.05 v/v) were prepared and elution was performed with a gradient flow of solvents from 50 to 100% with a flow rate of 1 mL min^{-1}. The fractions collected at the major peaks were tested for the antimicrobial activity and lyophilized.

2.7. FTIR and MALDI-TOF analysis

The active fractions were used for the structural elucidation by Shimadzu FTIR 8400 and spectrum was recorded from 4000 to 400 cm^{-1}, at an average of 20 scans was taken with the resolution of 4 cm^{-1}. The molecular mass of the purified fraction was determined by Matrix assisted laser desorption Ionization Time-of-Light (Applied Biosystems) and the mass to charge data was acquired on

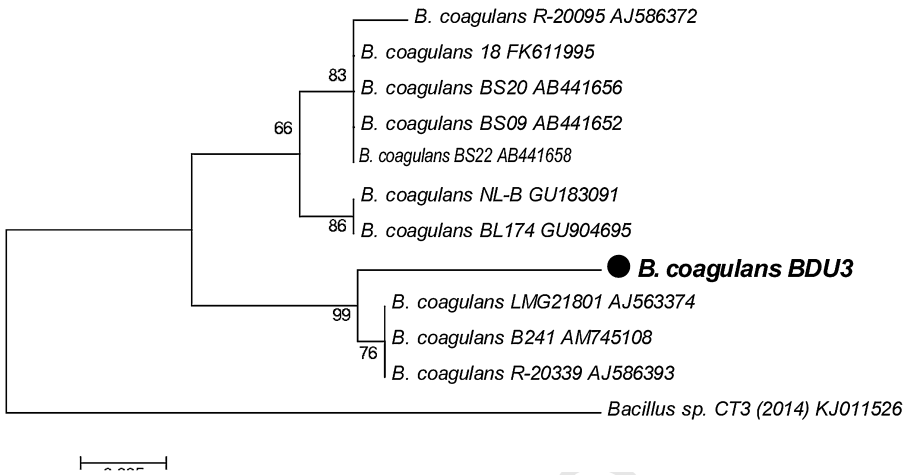

Fig. 1. Phylogenetic analysis of 16S rRNA sequence of *B. coagulans* BDU3.

400–2000 range, at an average of 30 scans were taken with 4 cm^{-1} resolution.

2.8. MIC of the purified bacteriocin

The purified bacteriocin stock solution was prepared and filter sterilized (0.22 μm pore size filter) prior to use. MIC was determined by microdilution method. Briefly, 24 h cultures of the panel of test microbes in THB were diluted in fresh broth medium to obtain an $OD_{600\,nm}$ 0.2. Equal volumes (100 μL) of bacterial culture and serially diluted bacteriocin were mixed into the wells of 96-well plates (Nunc Nalgene, USA). Wells with no bacteria or no bacteriocin served as controls. After an incubation of 24 h at 37 °C, bacterial growth was measured by recording $OD_{600\,nm}$. MIC values (μg/mL) of purified bacteriocin for each test organisms were determined as the lowest concentration at which no growth occurred. The MIC values were determined by three independent experiments.

2.9. Cell culture

HEK 293 (Human Embryonic Kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% (v/v) fetal bovine serum and 100 U/mL penicillin and streptomycin 100 U/mL at 37 °C and 5% CO_2. The viability of the test cells exceeded 99% prior to cytotoxicity assay as determined by exclusion of the dye trypan blue.

2.10. MTT assay

The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out to study the effect of bacteriocin on mammalian cells. HEK 293 cells (1×10^5 cells) were seeded in 96-well plates and incubated overnight at 37 °C and 5% CO_2. 200 μL of serial diluted purified bacteriocin (0.5–2.5 μg/mL) was added. Fresh DMEM and SDS were used as negative and positive control respectively. Plates were incubated for 24 h at 37 °C and 5% CO_2. The culture supernatants were removed and MTT solution (Promega, USA) was added to each well and the plates were incubated for 4 h at 37 °C and 5% CO_2. The MTT solution was removed and DMSO was added to dissolve formazan crystals. The absorbance at 540 nm was read on a Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). The percentage of viability was calculated as $A_T/A_C \times 100$; where A_T and A_C are the absorbances of treated and control cells, respectively.

2.11. Statistical analysis

Data were expressed as means ± SEM from three independent experiments. Significant differences were determined by Kruskal–Wallis test at level of significance of $P < 0.05$. The EC50 is defined as the concentration that caused 50% maximum effect and was calculated by regression analysis of the dose–response curves for MTT assay.

3. Results and discussion

3.1. Characterization of the isolate BDU3

The isolate BDU3 was characterized based on their morphology and biochemical characteristics. The isolate was phenotypically characterized as *Bacillus coagulans*. The isolate *Bacillus coagulans* BDU3 was a Gram-positive rod, positive for catalase, indole, Voges–Proskauer test. The optimal growth temperature was 45 °C and was found to be spore forming and motile. The isolate showed acid tolerance and survived at pH as low as 2.0 for at least 3 h but showed complete tolerance at pH values of 3 and 4. This showed the ability of the isolate to withstand human gastric pH and serve as a promising probiotics. The isolate exhibited bile tolerance at 0.1–0.2% above which the survival was greatly affected. Further, it can be proposed that the isolate can survive the challenging passage through the human GIT and can exert health benefits. The strain was characterized genotypically by 16S rRNA sequencing. The phylogenetic analysis showed the sequence to cluster with *Bacillus coagulans* and shared 99% sequence homology with *B. coagulans* LMG21801 (AJ563374), *B. coagulans* B241 (AM745108), *B. coagulans* R20339 (AJ586393) (Fig. 1).

Probiotic bacteria (*Lactobacillus*, *Bifidobacterium*, *Enterococci* and yeasts) are considered to be potential producers of metabolites and antimicrobial agents [23]. Thus they are considered economic and effective substitute for antibiotics. Though *Lactobacillus* and

Table 1
Indicator microorganisms used in the present study to evaluate the antimicrobial potential of *Bacillus coagulans* BDU3.

Microorganism	Diameter of the zone of inhibition (mm)
Bacillus cereus MTCC 430	7.6 ± 0.15
Staphylococcus aureus MTCC 3160	5.4 ± 0.11
Enterococcus sp. MTCC 9728	8.3 ± 0.5
Lactobacillus sp. MTCC 10093	3.2 ± 0.36
Micrococcus luteus MTCC 106	9.6 ± 0.6

The values are represented as mean ± SEM of three independent experiments.

Table 2
Effect of enzymes and detergents on the bioactive potential of CFS.

Enzyme/detergents	Specific activity (AU/mg protein)
Control	41
Proteases	
Proteinase K	ND
Pepsin	ND
Pronase E	ND
Other enzymes	
Catalase	25
Amylase	32.8
Lipase	28.7
Detergents	
SDS	ND
Tween 80	28.7
Triton X-100	28.7

ND – not detectable.

Bifidobacterium are widely used as probiotics, Bacillus has an added advantage due to its ability to sporulate and survive at ambient temperature [24]. Moreover *Bacillus* probiotics are marketed as a therapeutic drug with clinical evidence [25] and *Bacillus coagulans* has been recognized as GRAS (generally recognized as safe) by USFDA [26,27]. The phenotypic and genotypic characterization of the isolate BDU3 revealed it to be *Bacillus coagulans*.

3.2. Screening, characterization and identification of the antimicrobial compound of BDU3

The isolate *B. coagulans* BDU3 showed antimicrobial activity against *S. typhi* on the agar plates. The antimicrobial potency of the culture-free supernatant (CFS) as determined by well diffusion method showed inhibitory activity of CFS against a wide spectrum of pathogenic microorganisms (Table 1). *Bacillus* has been shown to produce antimicrobial peptides like amicoumacin A [28], polymyxin and polyfermenticin SCD [12] and Coagulin [29]. It has been generally accepted that bacteriocin exhibit specific activity against closely related organisms but studies has shown broad spectrum of antimicrobial potency for bacteriocin [30]. The strain BDU3 with the identified bacteriocinogenic potential has shown wide spectrum of antimicrobial activity against pathogens including *Staphylococcus aureus* and *Enterococcus* and also exhibited activity against closely related bacteria such as *Lactobacillus* and *Bacillus cereus*. The result of our present study supports the inhibitory activity of bacteriocin from *Bacillus* against *Staphylococcus*, for the control of mastitis in dairy farms [31].

In order to determine the nature of inhibitory substances in CFS, it was treated with enzymes and detergents. The antimicrobial activity of CFS was found to be sensitive to enzymes including Proteinase-K, Pepsin and Pronase E with no detectable levels of antimicrobial activity. The activity of CFS remained stable by treatment with catalase, amylase and lipase. Further the CFS was found to be sensitive to urea and EDTA with respective loss of 95 and 99% activity. In comparison, treatment with SDS/Tween 80/Triton X-100 induced a partial loss of activity. The action of the bacteriocin was slightly affected by detergents, which was similar to that observed in bacteriocin produced by *Lactobacillus lactis* [32]. The CFS was found to be significantly stable at temperatures of 30, 45 and 60 °C. Interestingly, the CFS showed 70% antimicrobial potency, withstanding temperature treatment of 100 and 121 °C for 30 and 15 min respectively. CFS activity remained stable in the pH range of 3–6 beyond which 25% reduction in activity was observed. The activity of bacteriocin from BDU3 was affected at alkaline conditions was in good agreement with previously characterized bacteriocins from *B. coagulans* [29]. As the CFS lost the activity by treatment with proteases, it can be predicted the active compound produced by *B. coagulans* BDU3 to be a protein, preferably bacteriocin (Table 2). Recently bacteriocin production is being increasingly considered as an important criterion for the selection of probiotic strains [33]. Several studies have shown the use of bacteriocin producing bacteria to be more effective to improve gut microflora and health compared to purified bacteriocin [34,35]. This is probably due to the degradation of purified bacteriocin by gut proteases and their inactivation before reaching the target site. On the other hand, bacteriocin produced in the intestine by Probiotic strains enhances direct interaction of sensitive pathogenic organisms [36,37].

Table 3
MIC of bacteriocin against the test organisms.

Microorganism	MIC of purified bacteriocin
Bacillus cereus MTCC 430	0.5 ± 0.26
Staphylococcus aureus MTCC 3160	1.0 ± 0.06
Enterococcus sp. MTCC 9728	0.75 ± 0.12
Lactobacillus sp. MTCC 10093	2.5 ± 0.19
Micrococcus luteus MTCC 106	0.75 ± 0.27

The values are represented as mean ± SEM of three independent experiments.

Then, we determined the MIC of the purified bacteriocin against the panel of test organisms. As reported in Table 3, the MIC values ranged from 0.5 to 2.5 μg/mL. The bacteriocin showed greater inhibitory activity against *Bacillus cereus* (0.5 μg/mL) followed by *Enterococcus* sp. (0.6 μg/mL). *Micrococcus luteus* was inhibited at a bacteriocin concentration of 2.5 μg/mL. Converse to the commercial antibiotics that have inhibitory activity at a very wide MIC range, the bacteriocin produced had a very narrow range of MIC (0.5–2.5 μg/mL).

3.3. Growth and bacteriocin production kinetics

Simultaneous measurement of the growth kinetics and bacteriocin production was studied. Detectable levels of bacteriocin activity were noted at 4 h of fermentation after which it reached the peak at 24 h. After 24 h the production plateaued till 36 h with subsequent decline. The maximum bacteriocin production and thus its activity were observed at the end of exponential growth phase of *B. coagulans* BDU3 (Fig. 2). Further characterization and purification of bacteriocin was achieved from the CFS obtained in the early stationary growth phase of *B. coagulans* BDU3.

3.4. Purification and characterization of bacteriocin

The CFS was obtained after 24 h of fermentation. The crude CFS was filtered through a 0.2 μm membrane and activity determined by disk diffusion method. Crude bacteriocin exhibited 44.50 AU/mg specific activity. The partially purified and dialyzed

Table 4
Summary of the purification steps of bacteriocin from the culture supernatant of *Bacillus coagulans* BDU3.

Fraction	Total Protein (mg)	Total activity (AU/mL)	Specific activity (AU/mg)	Increase in specific activity (fold increase)
Crude bacteriocin	314.6	70×10^5	44.50	1
Partially purified	56	27×10^5	192	4.3
Dialyzed sample	18.25	152×10^3	416.43	9.3
RP-HPLC	0.64	5.4×10^3	8437	189.59

Please cite this article in press as: K. Abdhul, et al., Int. J. Biol. Macromol. (2015), http://dx.doi.org/10.1016/j.ijbiomac.2015.06.005

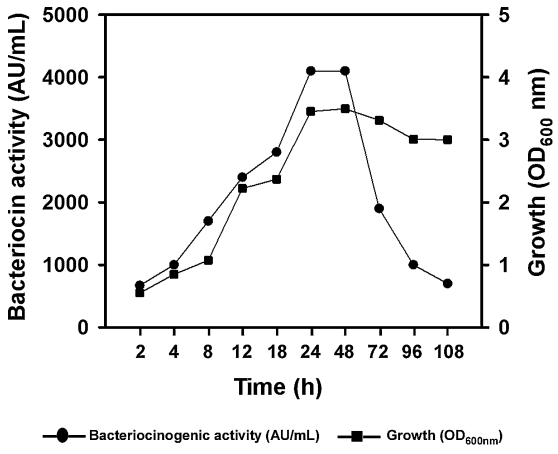

Fig. 2. Growth kinetics and bacteriocin production by *Bacillus coagulans* BDU3. *X*-axis: time (h). Y_1-axis: bacteriocin activity (AU/mL) and Y_2-axis: $OD_{600\,nm}$.

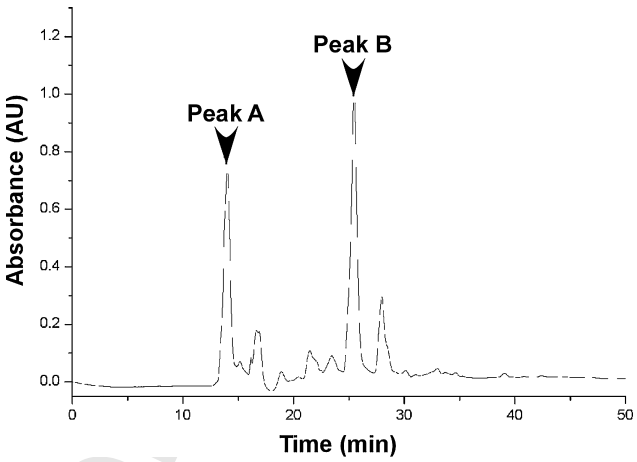

Fig. 3. Reverse phase-high performance liquid chromatogram of bacteriocins purified from *Bacillus coagulans* BDU3. *X*-axis: Time (min). *Y*-axis: Absorbance (AU). Elution of two predominant peaks (Peak A and Peak B) was obtained.

CFS (60% ammonium sulphate precipitation) showed 192 AU/mg and 416.43 AU/mg specific activities respectively (Table 4). The fractionation of the dialyzed preparation by RP-HPLC C18 column eluted two major peaks at retention time of 14.38 and 25.14 min respectively (Fig. 3). The fractions were collected individually at specified retention time and designated peak A and peak B respectively. The fractions collected at peak B showed an activity of ~8500 AU/mg and it was further characterized by FTIR and MALDI-TOF analysis.

3.5. Effect of bacteriocin on HEK 293 cells

The cytotoxicity effect of the purified bacteriocin was initially evaluated by using MTT assay. The results of the dose–response curves for MTT assay are presented in Fig. 4. Following exposure of HEK 293 cells to purified bacteriocin and SDS (positive control) caused a decrease of cell viability with increasing concentrations. When compared with SDS, bacteriocin caused significantly less

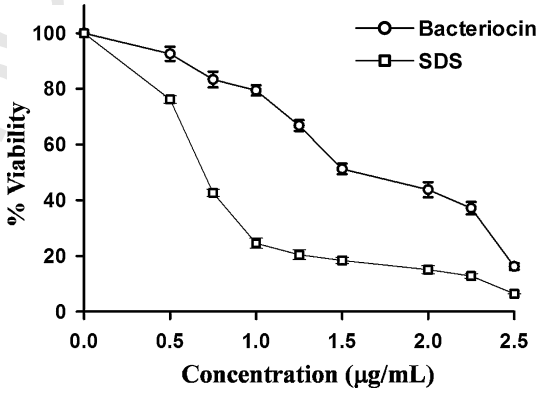

Fig. 4. Effect of purified bacteriocin on HEK 293 cells using MTT assay. The result represents mean ± SEM of three independent experiments.

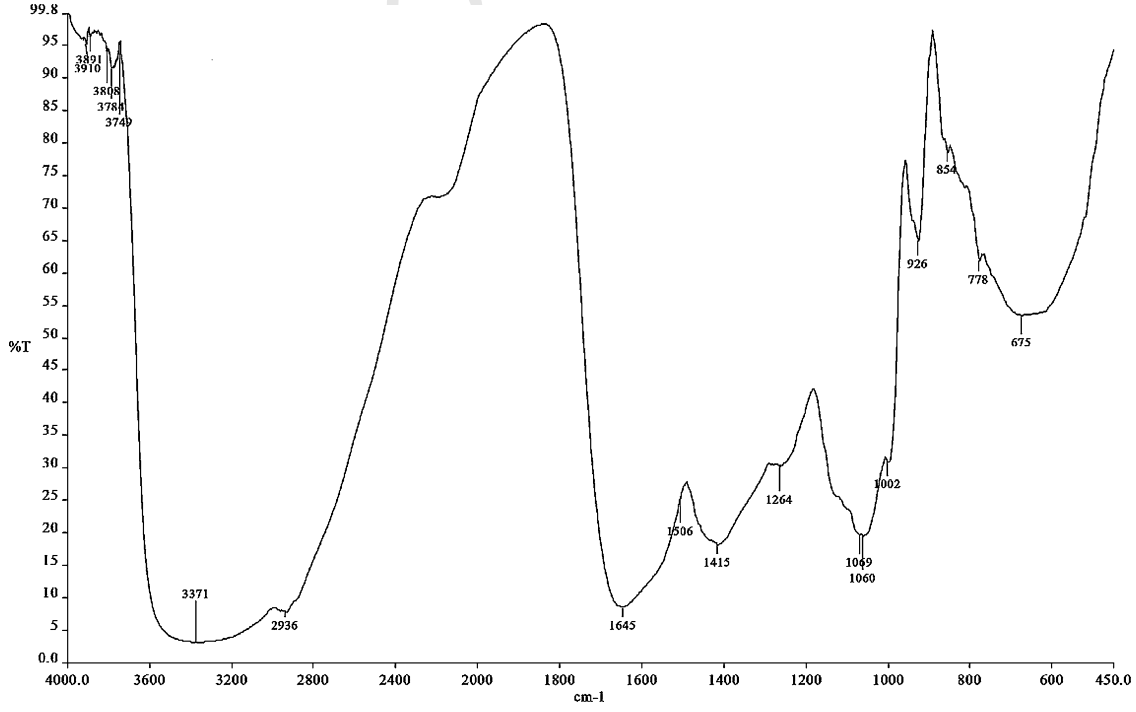

Fig. 5. FTIR spectrum of purified bacteriocins *Bacillus coagulans* BDU3.

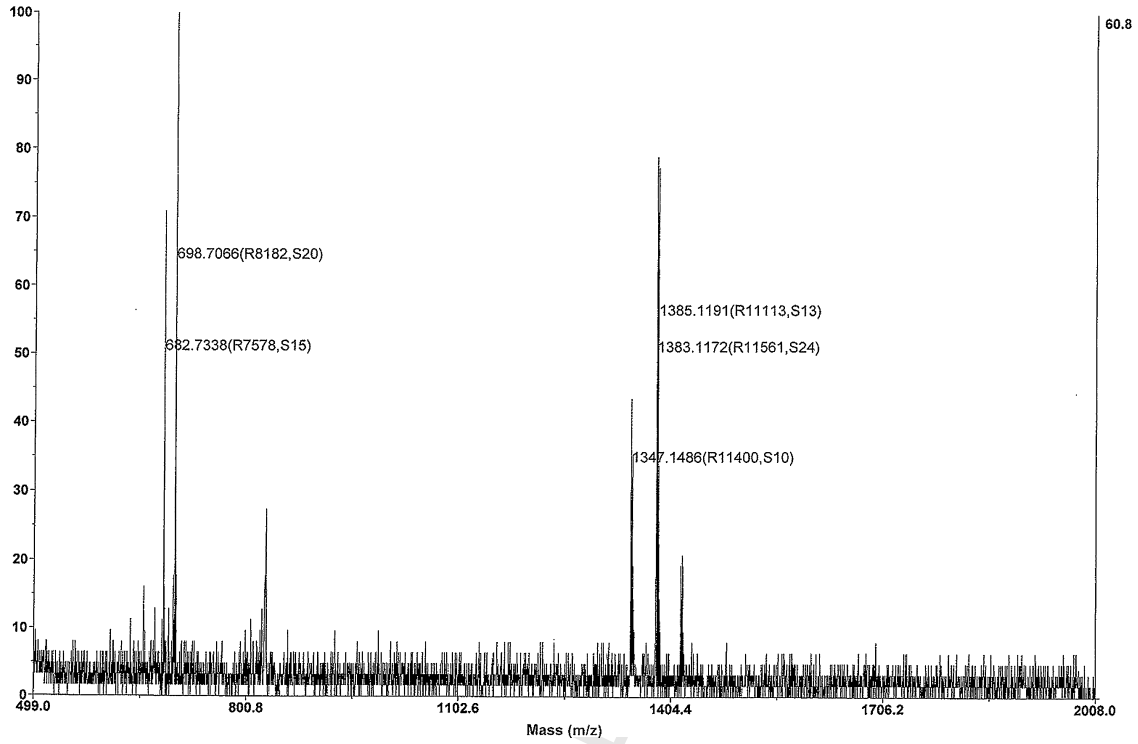

Fig. 6. MALDI-TOF spectrum of bacteriocins *Bacillus coagulans* BDU3.

($P < 0.05$) cytotoxicity at a concentration range of 0.5–2.5 μg/mL (MIC determined for a panel of microbes). Bacteriocin showed ~2 times lower EC50 values in MTT assay compared to SDS.

3.6. FTIR and MALDI-TOF

FTIR spectrum shows (Fig. 5) broad band between 3000 and 3600 cm^{-1} indicates – OH and NH stretching. The peaks at 1506 cm^{-1}, 1645 cm^{-1} (Gauzian amide bond) and band at 3371 cm^{-1} (Hydrogen bonded OH group) indicate the presence of peptide bonds. The spectral analysis indicates typical absorption peaks corresponding to N—H stretching of proteins and peptide bonds [38]. The peak at 1645 cm^{-1} associated with spectrum between 3500 and 3200 cm^{-1} indicates the amide functional group [39]. Absorption valley at 2936 cm^{-1} results from C—H stretching indicates the existence of aliphatic chain. The peak at 1415 cm^{-1} arises from the amide II band which results from the deformation of N—H bond combined with C—N Stretching molecule [40]. The peaks at 1645 cm^{-1} and 1506 cm^{-1} indicate the existence of amide I and amide II [41]. Fraction B obtained by RP-HPLC on C_{18} was analyzed by mass spectrometry and the molecular mass of the bacteriocin was 1.4 kDa (Fig. 6).

The MALDI-TOF analysis clearly points out that the peptide is a low molecular weight compound which is generally observed among bacteriocin. Le Marrec et al. (2000) [42] reported bacteriocin like compound coagulin, with a molecular mass of 4.6 kDa. On the other hand, Abada (2008) [43] reported antimicrobial compound with a molecular mass of 7.5 kDa from *Bacillus coagulans* with broad-spectrum activity against both Gram positive and negative bacterial and fungal pathogens. In contrast to this pediocin like bacteriocin with a molecular weight of 1.7 kDa from *Pediococcus pentosaceus* was reported [44]. Although low molecular weight coagulin has been previously reported, even though no peptides <1.5 kDa were reported from *Bacillus coagulans*. Increasing attention in natural antibiotics and food preservatives stimulate research in the field of bacteriocin as they can act as better alternatives to chemical based drugs.

4. Conclusions

The *B. coagulans* BDU3 isolated from Ngari exhibits the probiotic and bacteriocinogenic potential. To the extent of our knowledge this is the first report showing a low molecular weight antimicrobial peptide that is heat stable and with broad spectrum of antimicrobial activity. We appreciate the property of bacteriocins from *Bacillus coagulans* BDU3 as a potential bioactive candidate in food and pharmaceutical industries.

Acknowledgements

Authors greatly acknowledge Department of Biotechnology (DBT) and Department of Science and Technology (DST), Government of India for funding. We acknowledge Indian Institute of Science Education and Research (IISER), Pune for their help in MALDI-TOF studies. We also extent our thanks to the Vice-Chancellor, Bharathidasan University and the Principal and Management, Nandha College for the facilities provided.

References

[1] World Health Organization (WHO), Antimicrobial Resistance: No Action Today No Cure Tomorrow, WHO Press, 2011.
[2] M. Blaser, Antibiotic overuse: stop the killing of beneficial bacteria, Nature 476 (2011) 393–394.
[3] R.H. Perez, T. Zendo, K. Sonomoto, Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications, Microb. Cell Fact. 13 (Suppl. 1) (2014) S3.
[4] A. Gratia, Sur un remarquable exemple d'antagonisme entre deux souches de coilbacille, Comp. Rend. Soc. Biol. 93 (1925) 1040–1041.
[5] A.J. van Heel, M. Montalban-Lopez, O.P. Kuipers, A.J. Van Heel, Evaluating the feasibility of lantibiotics as an alternative therapy against bacterial infections in humans, Expert Opin. Drug Metab. Toxicol. 7 (2011) 675–680.

[6] P.D. Cotter, R.P. Ross, C. Hill, Bacteriocins – a viable alternative to antibiotics? Nat. Rev. Microbiol. 11 (2013) 95–105.
[7] L.H. Deegan, P.D. Cotter, C. Hill, P. Ross, Bacteriocins: biological tools for bio-preservation and shelf-life extension, Int. Dairy J. 16 (2006) 1058–1071.
[8] D. Drider, G. Fimland, Y. Héchard, L.M. McMullen, H. Prévost, The continuing story of class IIa bacteriocins, Microbiol. Mol. Biol. Rev. 70 (2006) 564–582.
[9] G. Fimland, L. Johnsen, B. Dalhus, J. Nissen-Meyer, Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action, J. Pept. Sci. 11 (2005) 688–696.
[10] R. Paul Ross, S. Morgan, C. Hill, Preservation and fermentation: past, present and future, Int. J. Food Microbiol. 79 (2002) 3–16.
[11] J. Delves-Broughton, Nisin as a food preservative, Food Aust. 57 (2005) 525–527.
[12] K.H. Lee, K.D. Jun, W.S. Kim, H.D. Paik, Partial characterization of polyfermenticin SCD, a newly identied bacteriocin of *Bacillus polyfermenticus*, Lett. Appl. Microbiol. 32 (2001) 146–151.
[13] W.C. Chan, B.W. Bycroft, M.L. Leyland, L.Y. Lian, G.C. Roberts, A novel post-translational modification of the peptide antibiotic subtilin: isolation and characterization of a natural variant from *Bacillus subtilis* A.T.C.C. 6633, Biochem. J. 291 (1993) 23–27.
[14] C.E. Shelburne, F.Y. An, V. Dholpe, A. Ramamoorthy, D.E. Lopatin, M.S. Lantz, The spectrum of antimicrobial activity of the bacteriocin subtilosin A, J. Antimicrob. Chemother. 59 (2007) 297–300.
[15] S. Riazi, R.E. Wirawan, V. Badmaev, M.L. Chikindas, Characterization of lactosporin, a novel antimicrobial protein produced by *Bacillus coagulans* ATCC 7050, J. Appl. Microbiol. 106 (2009) 1370–1377.
[16] K. Abdhul, M. Ganesh, S. Shanmughapriya, M. Kanagavel, K. Anbarasu, K. Natarajaseenivasan, Antioxidant activity of exopolysaccharide from probiotic strain *Enterococcus faecium* (BDU7) from Ngari, Int. J. Biol. Macromol. 70 (2014) 450–454.
[17] P. Logan, N.A. Turnbull, Bacillus and recently derived genera, in: Manual of Clinical Microbiology, ASM Press, Washington, DC, 1995, pp. 357–369.
[18] R.M. Smibert, N.R. Krieg, Methods for general and molecular bacteriology, in: K.N. P. Gerhardt, R.G.E. Murray, W.A. Wood (Eds.), Phenotypic Characterization, American Society for Microbiology, Washington, DC, 1994, pp. 607–654.
[19] R. Ben Salah, I. Trabelsi, R. Ben Mansour, S. Lassoued, H. Chouayekh, S. Bejar, A new *Lactobacillus plantarum* strain, TN8, from the gastro intestinal tract of poultry induces high cytokine production, Anaerobe 18 (2012) 436–444.
[20] U. Schillinger, F.K. Lücke, Antibacterial activity of Lactobacillus sake isolated from meat, Appl. Environ. Microbiol. 55 (1989) 1901–1906.
[21] C.B. Lewus, A. Kaiser, T.J. Montville, Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat, Appl. Environ. Microbiol. 57 (1991) 1683–1688.
[22] Y. Yamamoto, Y. Togawa, M. Shimosaka, M. Okazaki, Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* Strain RJ-11, Appl. Environ. Microbiol. 69 (2003) 5746–5753.
[23] M.C. Collado, S. Delgado, A. Maldonado, J.M. Rodríguez, Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR, Lett. Appl. Microbiol. 48 (2009) 523–528.
[24] H.A. Hong, H.D. Le, S.M. Cutting, The use of bacterial spore formers as probiotics, FEMS Microbiol. Rev. 29 (2005) 813–835.
[25] M.E. Sanders, L. Morelli, T.A. Tompkins, Sporeformers as human probiotics: bacillus, and brevibacillus, Compr. Rev. Food Sci. Food Saf. 2 (2003) 101–110.
[26] S. Farmer, A.R. Lefkowitz, Absorbent product containing absorbent structure and Bacillus coagulans, US Patent No. 6716435 B1.
[27] R. Sen, K.S. Babu, Modeling and optimization of the process conditions for biomass production and sporulation of a probiotic culture, Process Biochem. 40 (2005) 2531–2538.
[28] I.V. Pinchuk, P. Bressollier, B. Verneuil, B. Fenet, I.B. Sorokulova, F. Megraud, M.C. Urdaci, In vitro anti-*Helicobacter pylori* activity of the probiotic strain *Bacillus subtilis* 3 is due to secretion of antibiotics, Antimicrob. Agents Chemother. 45 (2001) 3156–3161.
[29] B. Hyronimus, C. Le Marrec, A. Hadj Sassi, A. Deschamps, Acid and bile tolerance of spore-forming lactic acid bacteria, Int. J. Food Microbiol. 61 (2000) 193–197.
[30] R.W. Jack, J.R. Tagg, B. Ray, Bacteriocins of gram-positive bacteria, Microbiol. Rev. 59 (1995) 171–200.
[31] H. Abriouel, C.M. Franz, N. Ben Omar, A. Gálvez, Diversity and applications of *Bacillus bacteriocins*, FEMS Microbiol. Rev. 35 (2011) 201–232.
[32] G. Rajaram, P. Manivasag, B. Thilagavathi, A. Saravanakum, Purification and characterization of a bacteriocin produced by *Lactobacillus lactis* isolated from marine environment, Adv. J. Food Sci. Technol. 2 (2010) 138–144.
[33] A. Dobson, P.D. Cotter, R. Paul Ross, C. Hill, Bacteriocin production: a probiotic trait? Appl. Environ. Microbiol. 78 (2012) 1–6.
[34] S. Cruchet, M.C. Obregon, G. Salazar, E. Diaz, M. Gotteland, Effect of the ingestion of a dietary product containing *Lactobacillus johnsonii* La1 on *Helicobacter pylori* colonization in children, Nutrition 19 (2003) 716–721.
[35] D. Lesbros-Pantoflickova, I. Corthésy-Theulaz, A.L. Blum, *Helicobacter pylori* and probiotics, J. Nutr. 137 (2007) 812S–818S.
[36] L. De Vuyst, F. Leroy, Bacteriocins from lactic acid bacteria: production, purification, and food applications, J. Mol. Microbiol. Biotechnol. 13 (2007) 194–199.
[37] O. Gillor, A. Etzion, M.A. Riley, The dual role of bacteriocins as anti- and probiotics, Appl. Microbiol. Biotechnol. 81 (2008) 591–606.
[38] K. Maquelin, C. Kirschner, L.P. Choo-Smith, N. van den Braak, H.P. Endtz, D. Naumann, G.J. Puppels, Identification of medically relevant microorganisms by vibrational spectroscopy, J. Microbiol. Methods 51 (2002) 255–271.
[39] L.B. Benitez, R.V. Velho, M.P. Lisboa, L.F.D.C. Medina, A. Brandelli, Isolation and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* LBM5006, J. Microbiol. 48 (2010) 791–797.
[40] K. Ajesh, S. Sudarslal, C. Arunan, K. Sreejith, Kannurin, a novel lipopeptide from *Bacillus cereus* strain AK1: isolation, structural evaluation and antifungal activities, J. Appl. Microbiol. 115 (2013) 1287–1296.
[41] H. Fabian, C. Schultz, D. Naumann, O. Landt, U. Hahn, W. Saenger, Secondary structure and temperature-induced unfolding and refolding of ribonuclease T1 in aqueous solution. A Fourier transform infrared spectroscopic study, J. Mol. Biol. 232 (1993) 967–981.
[42] C. Le Marrec, B. Hyronimus, P. Bressollier, B. Verneuil, M.C. Urdaci, Biochemical and genetic characterization of coagulin, a new antilisterial bacteriocin in the pediocin family of bacteriocins, produced by *Bacillus coagulans* I(4), Appl. Environ. Microbiol. 66 (2000) 5213–5220.
[43] E.A.E. Abada, Isolation and characterization of a antimicrobial compound from *Bacillus coagulans*, Animal Cells Syst. (Seoul) 12 (2008) 41–46.
[44] P.K. Singh, S. Sharma, A. Kumari, S. Korpole, A non-pediocin low molecular weight antimicrobial peptide produced by *Pediococcus pentosaceus* strain IE-3 shows increased activity under reducing environment, BMC Microbiol. 14 (2014) 226.