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Research Article

Partial Characterization of an Anti-*Candida albicans* Bacteriocin Produced by a Marine Strain of *Bacillus* sp., Sh10

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Abstract: The bacteriocin-producing strain *Bacillus* sp., Sh10, isolated from the marine environment, exhibited a broad spectrum of antimicrobial activity against different food spoilage and human pathogens, with a maximum inhibitory activity against *Candida albicans*. The inhibitory compound was sensitive to trypsin but resistant to proteinase K, lysozyme, lipase and α-amylase. It was heat-stable and remained its activity after autoclaving. In addition, the antimicrobial substance demonstrated striking stability at low temperatures (4 and -20°C) for up to one year and retained its activity in a wide pH range from 2 to 11. It was also stable and active in the presence of different surfactants, solvents and heavy metals. Analysis of the partially purified bacteriocin by SDS-PAGE showed an apparent molecular weight of ~11 KDa. This study reveals a remarkable potential of this bacteriocin to be used as a food preservative.

Keywords: *Bacillus* sp., Sh10, bacteriocin, *Candida albicans*, marine clam, partial characterization

INTRODUCTION

Fungal and yeast contamination issues in several food products cause health problems and economic losses due to the spoilage and decomposition of food products, the deterioration of organoleptic properties and the potential production of mycotoxins or allergenic conidia (Hussein and Brasel, 2001). To overcome this problem, there is a need to control and intercept their growth in different food products. Chemical additives (fungicides) are not suitable to address this problem, due to their harmful side-effects. Nowadays, natural preservatives with no side effects are much sought-after. Among these compounds, bacteriocins are the most suitable substances to reduce the need for chemical preservatives in food and minimize the intensity of food processing techniques, contributing to the production of healthier foods (Osmanagaoglu *et al*., 2011).

Bacteriocins are inhibitory peptides or proteins produced by a group of bacteria, with a bacteriocidal effect on a different group of microorganisms that are closely related to the producer (Jack *et al*., 1995). Bacteriocins are heterogeneous compounds, which vary in molecular weight, biochemical properties, activity spectra and mechanism of action (Klaenhammer, 1988). Similar to Lactic Acid Bacteria (LAB), which are the most-studied bacteriocin-producing microorganisms, *Bacillus* spp. also produce different antimicrobial peptides with a diverse array of chemical structures (Xie *et al*., 2009; Abriouel *et al*., 2010). An increasing number of compounds with antimicrobial activity have been found to be produced by a variety of organisms present in the marine environment (Selvin *et al*., 2004; Romanenko *et al*., 2008); however, only few studies have focused on the ability of marine bacteria to produce bacteriocins. Therefore, in the present study, we report the isolation and partial characterization of heat-stable bacteriocin produced by *Bacillus* sp., strain Sh10 isolated from the marine clam, with a strong inhibitory activity against the food spoilage pathogen, *Candida albicans*.

MATERIALS AND METHODS

Bacterial strains and culture conditions: The bacteriocin-producing strain Sh10 was isolated from the carpet clam (*Paphia textile*) using marine agar medium and was cultured in optimized medium containing inorganic salts (Pridham and Gottlieb, 1948), 2% tryptone, 1% glucose and 2% NaCl. The culture medium was adjusted to pH 8 and bacteria were incubated aerobically for 30 h at 30°C. *Candida albicans* was the indicator strain. Other strains used in this study are listed in Table 1. *C. albicans* was grown in TSB medium at 30°C, whereas other strains were incubated in nutrient broth medium at 37°C. All strains were kept as stocks at 80°C in 40% glycerol.

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Table 1: Antimicrobia spectrum of bacteriocin produced by strain *Bacillus* sp., Sh10

Indicator bacteria	Bacteriocin activity (AU/mL)
Candida albicans	1200
Bacillus subtilis ATCC11774	-
Bacillus thuringiensis ATCC10792	400
Sthapylococcus aureus ATCC11632	200
MRSA N32064	500
Pseudomonas aeruginosa ATCC27853	-
Escherichia coli ATCC10536	-
Enterobacter aerogenes ATCC51697	-
Enterobacter faecalis ATCC14506	-
Serratia marcescens ATCC13880	-
Salmonella typhimurium ATCC51312	-
Salmonella mutan ATCC25175	-
Klebsiella pneumonia ATCC10031	-
Aeromonas hydrophila Wbf314	500
Aeromonas hydrophila Ctt6	500
Vibrio parahaemolyticus ATCC17802	500
Vibrio parahaemolyticus VWPAm1	500
Vibrio alginolyticus VWMPLps2	-
Vibrio vulnificus VPMAm1	-
Vibrio harveyi VSPAm1	-
Alcaligenes faecalis	-
Proteus mirabilis	-

-: Indicator strain not inhibited by bacteriocin

Bacteriocin activity assay: Bacteriocin activity of the strain Sh10 was detected by the cross-streaking method (Poh *et al*., 1983) and confirmed by the spot-on-lawn method (Seuk-Hyun and Cheol, 2000). For the cross-streaking method, the present isolate was inoculated in a 1-cm streak onto marine agar plates and was incubated at 30°C. After 24 h, plates were exposed to chloroform vapor for 30 min to kill the bacteria. The plates were then exposed to air in a laminar flow cabinet for 1 h to allow evaporation of the remaining chloroform. Cultures of the test strains, which contained 1×10^8 CFU/mL cells, were streaked perpendicular to the original line of growth on each producer plate. All plates were again incubated at 30°C overnight to observe the clear zone around the producer cultures. For the spot-on-lawn method, bacteria Sh10 was grown in optimized medium as mention above. After 30 h, the culture was centrifuged at 12,000 rpm for 30 min at 4°C and then filtered through a cellulose acetate filter (0.22 μm) to remove residual cells. Next, 10 μL of cell-free supernatant was spotted onto the surface of Muller Hintone Agar overlaid with 1×10^8 CFU/mL cells of the indicator organisms and incubated at 37°C for 24 h, except for *C. albicans*, which was incubated at 30°C. The amount of bacteriocin production was calculated as an Arbitrary Unit (AU) per mL, defined as the reciprocal of the highest serial two-fold dilution that showed a clear zone of growth inhibition of the indicator strain (Hoover and Harlander, 1993).

Bacterial identification: Identification of the producer strain was performed by 16S rRNA sequencing, fatty acid analysis, G+C (%mol) content and carbon-source utilization. Genomic DNA extraction was carried out using the Wizard Genomic DNA Purification Kit (Promega). The PCR amplification of the 16S rRNA gene was performed using the oligonucleotide primers: forward (5 'AGA GTT TGA TCC TGG CTC AG 3') and reverse (5 'GGT TAC CTT GTT ACG ACT 3'). The PCR program, which was performed in a heat thermal minicycler (MJ Research, USA) was as follows: Initial denaturation was carried out for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 90 sec, with a further 10 min extension at 72°C, using the UniversAll^{TM} tissue PCR kit. The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) according to the manufacturer's instructions. The resulting sequence (1,500 bp) of strain Sh10 was compared with that in GenBank using the program BLASTn (Altschul *et al*., 1997). The phylogenetic tree of related sequences based on BLAST sequence similarity was constructed using the MEGA version 4 program (Tamura *et al*., 2007) after multiple alignments of data by CLUSTAL-W (Thompson *et al*., 1994) for the assignment of close relationships at the species level. Nucleotide sequence similarities and DNA gaps were calculated and edited using BIOEDIT software (Hall, 1999). Bootstrap analysis (1,000 re-samplings) was used to evaluate the topology of the neighbor-joining tree (Felsenstein, 1985). The G+C content (mol%) of strain Sh10 was determined using the melting temperature method as described by Marmur and Doty (1962) and Mandel *et al*. (1970). Determination of the fatty acid profile was performed by the method of Folch *et al*. (1957) using gas chromatography-mass spectroscopy. Carbon-source utilization was carried out using a Biolog GP2 microplate according to the manufacturer's instructions.

Identification using Scanning Electron Microscopy (SEM): Scanning electron microscopy was performed on a 24-h culture of strain Sh10 grown on marine agar to assess the morphological characteristics. Colonies on the plate were cut within a 1×1 cm area and fixed in 0.2% glutaraldehyde overnight. Serial dehydration from 30 to 100% was performed, followed by Critical Point Drying (CPD). Before viewing, the sample was sputtered with gold and viewed using a Philips XL 300 SEM.

Stability of bacteriocin to enzymes, heat and pH: Cell-free supernatant was used to determine the resistance of bacteriocin to enzymes, pH and temperature. The aliquots were subjected to different temperatures: 40, 60 and 80°C for 30 min, 100°C for 15 and 30 min, 121°C for 20 min, or 4°C and -20°C for up to 1 year, respectively. To test the stability of bacteriocin at different pHs, aliquots were adjusted to a pH range of 2.0-11.0 using 5 M HCl or NaOH and were incubated at room temperature for 2 h. To examine the sensitivity of bacteriocin to different enzymes, cell-free supernatant was pretreated with 1 mg/mL final concentration of enzymes such as proteinase K, trypsin, lysozyme, lipase and α-amylase at room temperature for 2 h. Untreated bacteriocin (1,200 AU/mL) was used

as a control and residual activity was tested using the same method as described already.

Effect of different surfactants, solvents and metal ions on bacteriocin activity: The effect of surfactants on bacteriocin was tested by adding Tween 20 and 80 (1% final concentration v/v), SDS (1% final concentration w/v), EDTA and urea at a final concentration of 2.5 mM and 6 M, respectively, to the cell-free supernatant. In a separate experiment, the effect of metal salts on bacteriocin activity was tested by adding $NiSO_4.6H_2O$, $MgSO_4$, $FeSO_4.6H_2O$, $Co(NO_3)_2.6H_2O$, $CuSO_4$, $ZnSO_4$ or $MnCl_2$, (1 mM final concentration) to the culture supernatant. To test the resistance of bacteriocin to different solvents, cell-free supertatants were treated with organic solvents, including ethanol, methanol, acetone, diethylether, 2-propanol, n-hexane, chloroform and isoamyl alcohol at a final concentration of 25% (v/v). Untreated bacteriocin (1, 200 AU/mL) was used as a positive control. The organic solvents, metal salts and detergents at the same concentrations were also used as negative controls. All the samples were incubated at room temperature for 2 h and residual activity was tested using the same method described earlier.

Partial purification of bacteriocin: The culture supernatant was used as the starting material for protein extraction. Ammonium sulfate was slowly added to a final concentration of 80% (w/v) and the mixture was continually stirred at 4°C for 24 h. The precipitate was collected by centrifugation at 12,000 rpm for 30 min and was dissolved in 10 mM phosphate buffer.

Bacteriocin molecular weight estimation by SDS-PAGE: The direct detection of bacteriocin on SDS-

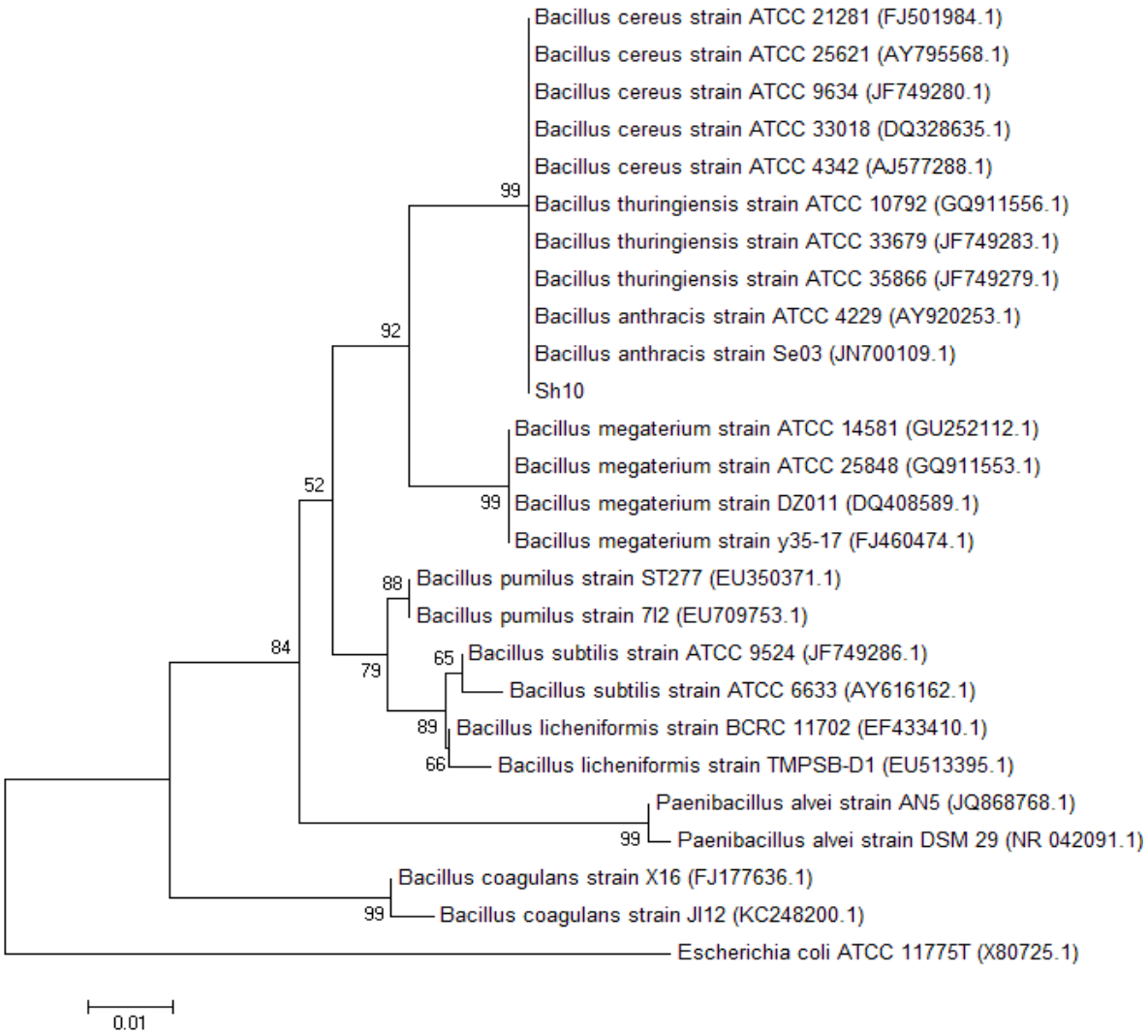

Fig. 1: Phylogenetic genetic relationship of *Bacillus* strain Sh10 to closely related bacteria based on neighbor joining tree analysis of 16S rRNA gene sequencing. A sequence of *Escherichia coli* was used as the out-group

polyacrylamide gels was conducted as described by Laemmli (1970). The activity of ammonium sulfate-precipitated bacteriocin preparation from strain Sh10 was determined by SDS-polyacrylamide (12%) gels. The gel was vertically cut into two halves; the first half was stained with Coomassie blue R-250 to view the protein bands, while the other half was washed several times in sterile distilled water and overlaid with Muller Hintone soft agar seeded with 1×10^8 CFU/mL cells of the indicator organism. The gel was incubated at 30°C for 24 h and was examined for a zone of growth inhibition caused by bacteriocin within the gel. A pre-stained broad-range molecular mass marker (GeneDirex) was used to define the molecular mass of bacteriocin.

RESULTS

From 30 bacteria isolated from marine clams, isolate Sh10 was found to produce bacteriocin with a broad inhibitory activity by cross-streaking and spot-on-lawn methods. Comparative 16S rRNA gene sequencing analysis of this strain revealed 95% similarity with *B. cereus*, *B. thuringiensis* and *B. anthrasis* (Fig. 1). The DNA G+C mol% content of the present strain was 36.12 mol%. Twelve different fatty acids were detected in the cellular fatty acid profile of strain Sh10, which was comprised of 12:0 iso (1.42%), 12:0 (0.43%), 13:0 iso (9.86%), 13:0 anteiso (2.85), 14:0 iso (5.37%), 14:0 (3.99%), 15:0 iso (28.67%), 15:0 anteiso (4.45%), 16:0 iso (9.22), 16:0 (11.76), 17:0 iso (4.54%) and 17:0 anteiso (1.17%) fatty acids. Isolate Sh10 was able to use 59 out of 96 types of carbon sources after 24 h, which was 85% similar to the sources used by *B. cereus* and *B. thuringiensis*. The image obtained from scanning electron microscopy of this strain is presented in Fig. 2.

The culture supernatant of this strain inhibited several types of Gram-positive bacteria such as *Staphylococcus aureus*, Methicillin-resistant *S. aureus* and *B. thuringiensis*. However, the culture filtrate did not inhibit Gram-positive *B. subtilis*. Among the Gram-negative bacteria tested, only *Vibrio parahaemolyticus* and *Aeromonas hydrophilia* were inhibited. The culture supernatant strongly inhibited the yeast *C. albicans* with maximum inhibitory activity, compared to other tested strains (Table 1).

Among different enzymes tested for bacteriocin sensitivity, proteinase k, lysozyme, lipase and α amylase did not affect the antimicrobial activity of bacteriocin, but complete inactivation was observed after treatment with trypsin. Bacteriocin activity remained after heat treatment at 40, 60, 80 and 100°C, respectively for 30 min and remained stable after

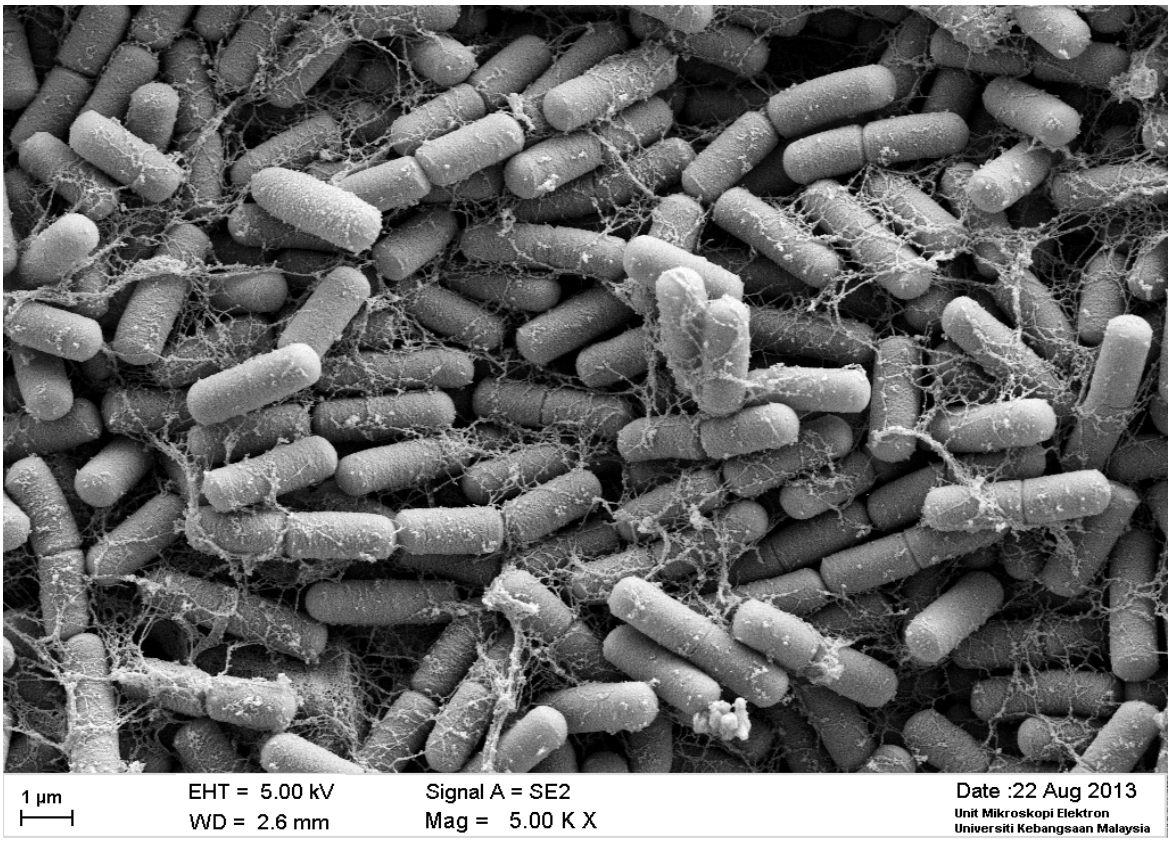

Fig. 2: Scanning electron microscopy of strain *Bacillus* sp., Sh10

Table 2: Effect of different treatment on cell-free supernatant of *Bacillus* sp., SHh10 against *C. albicans*

Treatment and condition	Residual bacteriocin activity (AU/mL)
Control	1200
Enzyme (2 h, room temperature)	
Trypsin (1 mg/mL)	0
Proteinase k	1200
Lysozyme	1200
Lipase	1200
α-amylase	1200
Temperature	
40°C (30 min)	1200
60°C (30 min)	1200
80°C (30 min)	1200
100°C (15 min)	1200
100°C (30 min)	1200
121°C (20 min)	1200
4°C (1 year)	1200
-20°C (1 year)	1200
PH (2 h, room temperature)	
2.0	1000
3.0	1200
4.0	1200
5.0	1200
6.0	1200
7.0	1200
8.0	1200
9.0	1200
10.0	800
11.0	800
Surfactant (2 h, room temperature)	
Tween 20 (1% v/v)	1200
Tween 80 (1% v/v)	1200
EDTA (2.5 mM)	1200
Urea (6 M)	1200
SDS (1% w/V)	1200
Solvent (25% v/v, 2 h, room temperature)	
Methanol	1200
Acetone	400
Diethylether	400
N-hexane	1200
2-propanol	1200
Isoamylalcohol	1200
Chloroform	1200
Ethanol	1200
Heavy metal (1 mM, 2 h, room temperature)	
$Mgso_4$	1200
$Niso_4.6H_2o$	1200
$Co\ (No_3)_2.6H_2o$	1200
$Feso_4.6H_20$	1200
$Cuso_4$	1200
$Mncl_2$	1200
$Znso_4$	1200

autoclaving (121°C, 15 psi for 20 min). These findings showed that an inhibitory compound produced by this strain is a heat-stable proteinaceous compound. Bacteriocin remained active at low temperatures of 4°C and -20°C for up to 1 year. It was also stable after incubation for 2 h at pH values from 3-9, but showed slightly decreased activity at pH values of 2, 10 and 11, respectively. Bacteriocin activity did not alter when exposed to different surfactants and was resistant to different solvents, except for diethyl ether and acetone, which strongly decreased the bacteriocin activity. No change in bacteriocin activity was found following treatment with different heavy metals (Table 2).

The molecular weight of crude bacteriocin produced by strain Sh10 was determined by SDS-PAGE electrophoresis. As shown in Fig. 3, the bacteriocidal activity of the bacteriocin crude extract from this strain had a molecular weight of about 11 KDa.

DISCUSSION

The present study demonstrates that the marine environment is a good source for the isolation of bacteriocin with a strong inhibitory effect against different pathogenic microorganisms. Here, we report a bacteriocin-producing bacterium that was isolated from the marine clam. Based on 16s rRNA gene sequencing, we demonstrated that strain Sh10 belongs to the genus *Bacillus* and to the *B. cereus* group, with 95% similarity to *B. cereus*, *B. thuringiensis* and *B. anthrasis*. These three species are genetically very closely related members of the *B. cereus* group, which has led to the suggestion that they be considered as a single species (Helgason *et al*., 2000). The G+C content of the present strain was 36.12 mol%, which is in the range of 32 to 69 mol% for the genus *Bacillus* (Priest, 1981). The fatty acid profile of the present strain was very close to that of *B. cereus*, *B. thuringiensis* and *B. anthrasis*. There is a remarkable similarity in the fatty acid composition of these closely related species and it is difficult to distinguish them based on their fatty acid profile (Kaneda, 1968). However, the carbon-source utilization profile of strain Sh10 showed a higher similarity to *B. cereus* and *B. thuringiensis*, with 85% homology. Based on this finding, it can be assumed that this strain is a new candidate member of the *B. cereus* group. In some cases, it is difficult to differentiate *Bacillus* species and a large number of phenotypic tests must be performed to distinguish between them; however, sometimes only a single feature separates them (Logan and Berkeley, 1984; Drobniewski, 1993). The bacteriocin produced by the present isolate showed high inhibitory activity against different clinical and food-borne pathogens and its antimicrobial property is comparable to that of a broad range of bacteriocins associated with other Bacillus spp. such as cerein 7, isolated from *B. cereus* BC7 (Oscariz and Pisabarro, 2000), colagolin, produced by *B. coagulans* 14 (Hyronimus *et al*., 1998) and bacteriocin produced by *B. cereus* A8 (Bizani, and Brandelli, 2002). The highest inhibitory activity was demonstrated against *C. albicans*. The inhibitory activity of yeast by bacteriocins produced by *Bacillus* species was reported for *B. licheniformis* MKU3 (Kayalvizhi and Gunasekaran, 2008) and *B. brevis* (Mah *et al*., 2001). In addition, the test strain exhibited strong inhibition against the two important causes of food-borne gastroenteritis pathogens, *V. parahaemoliticus* and *A. hydrophilia*. The inhibitory effect of bacteriocin

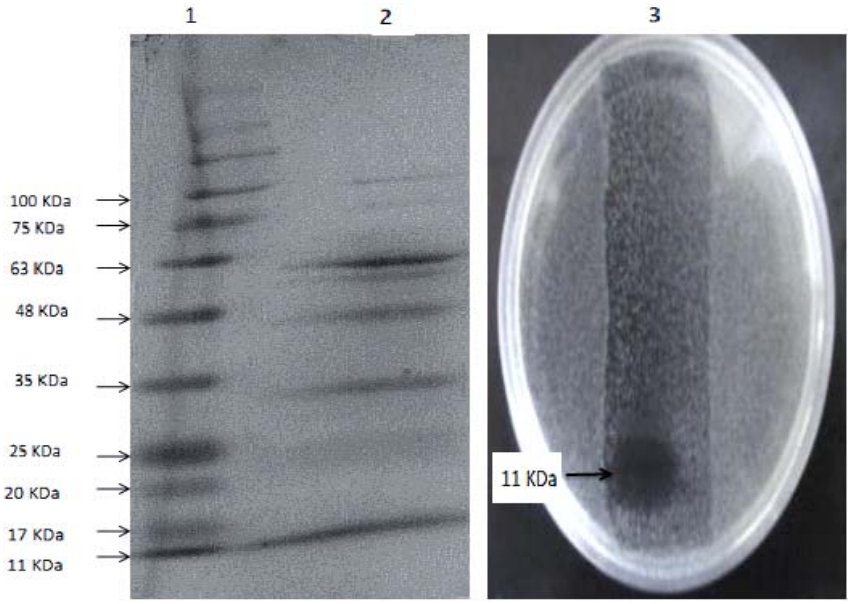

Fig. 3: SDS-PAGE analysis of bacteriocin produced by strain Sh10 after precipitation with ammonium sulfate
Lane 1: Molecular weight marker; Lane 2: Bacteriocin extract; Lane 3: SDS-PAGE gel showing the zone of inhibition against *C. albicans* by the bacteriocin

against food-borne pathogens has raised great concern for their application in the food industry as a preservative (Galvez *et al*., 2008).

The complete inactivation of the cell-free supernatant after treatment with trypsin supports the proteinaceous nature of the inhibitory compound. Treatment of bacteriocin with proteinase K did not affect its activity. The sensitivity of bacteriocin to trypsin was also observed in bacteriocins produced by *B. subtilis* 14B (Hammami *et al*., 2009) and *B. licheniformis* MKU3 (Kayalvizhi and Gunasekaran, 2008). Lipase, α amylase and lysozyme also did not affect bacteriocin activity, suggesting the absence of lipid, polysaccharide or glycoside moieties in this bacteriocin.

Bacteriocin was active both in the presence and absence of oxygen and exhibited great stability across a wide range of pH values (3.0-9.0), with a considerable reduction in activity only at a pH of 2, 10 and 11, respectively as reported previously (Mandal *et al*., 2008; Sarika *et al*., 2010). Bacteriocin stability in alkaline conditions is now gaining more attention in the food industry, because the pH of several food products varies from natural to alkaline. It has been reported that nisin is the only commercial bacteriocin used as a food supplement at acidic pH, whereas it is unstable at alkaline pH (Liu and Hansen, 1990).

The present bacteriocin was completely stable to heat treatment and retained activity after autoclaving for 20 min at 121°C. Its activity was also stable under cooling and freezing temperatures for up to one year. Fangio and Fritz (2013) also obtained similar results in a storage study of CBLC produced by *B. subtilis*. The notable feature of the heat resistance and long storage at low temperatures of this bacteriocin supports its potential use as a food preservative in different processes such as drying, pasteurization, freezing and refrigeration, without a reduction in its antimicrobial activity.

Surprisingly, the bacteriocin remained unaffected by heavy metals and solvents, except for 25% acetone and diethylether, thus confirming a high resistance and supporting its potential commercial application. It also retained complete activity following exposure to surfactants. Similar results were recorded for bacteriocins isolated from *Lactococcus lactis* W8 (Mitra *et al*., 2005) and *Pediococcus acidilactici* (Albano *et al*., 2007).

SDS-PAGE analysis of partially purified bacteriocin of the strain Sh10 demonstrated that the 11 KDa band was responsible for the *C. albicans* inhibition. The estimated molecular weight of the present bacteriocin was somewhat similar to thuricin (11.6 KDa) produced by *B. thuringiensis* BMG1.7 (Cherif *et al*., 2001) and entomocin 9 (12.4 KDa) from *B. thuringiensis* ssp. Entomocidus HD9 (Cherif *et al*., 2003) but higher than cerein 7 (3.94 KDa) produced by *B. cereus* BC7 (Oscariz *et al*., 1999), coagulin (4.61 KDa) from *B. coagulans* I4 (Le Marrec *et al*., 2000) and bacthuricin F4 (3.16 KDa) from *B. thuringiensis* ssp. kurstaki strain BUPM4 (Kamoun *et al*., 2005).

CONCLUSION

This investigation has ascertained that bacteriocin produced by a new bacterial strain, *Bacillus* sp., Sh10,

possesses a broad spectrum of inhibitory activity. The stability of bacteriocin to heat and a wide range of pH, different solvents, heavy metals and detergents, is considered to be very important and make it an interesting candidate for a bio-preservative on an industrial-scale. Work is currently in progress to purify and determine the amino acid sequence of this bacteriocin as well as its mode of action.

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