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Purification and Characterization of an Antibacterial Peptide (Bacteriocin) Produced By *Weissella confusa* AJ79

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Abstract: *A non-pathogenic lactic acid bacterium, Weissella confusa AJ79, isolated from uttapam batter fermented supplementing with Piper betle L. leaves, was screened and found to produce multiple antibacterial peptides (bacteriocins). An antibacterial peptide (ABP), BAC79, released in cell-free supernatant was purified by SP-Sepharose fast flow cation exchange chromatography, C18 Sep-pak cartridge hydrophobic interaction chromatography, and Sephadex G-25 (superfine) gel permeation chromatography. The molecular weight of the BAC79 was about 5 kDa analyzed using Tris-tricine SDS-PAGE. The peptide mass fingerprint data analysis of the trypsin-digested bacteriocin BAC79 using MASCOT server had shown that it was an unknown ABP as the de novo MS/MS amino acid sequence analysis had no homology with any reported bacteriocin or ABP. The ABP BAC79 was found to be active and stable at different pH and temperature; sensitive to proteolytic enzymes, stable with different detergents and inhibited a number of wide varieties of pathogenic microorganisms. It was a membrane active peptide which inhibited the growth of Listeria monocytogenes by disrupting its cell membrane. Based on its characteristics, the bacteriocin BAC79 can be brought under class II of bacteriocins. The ABP BAC79 can be used for the management of pathogens in medicals for foodborne pathogens and in food safety as food additives for food spoilage microorganisms.*

Keywords: *Bacteriocins; lactic acid bacteria; peptides; proteomics; preservatives*

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I. Introduction

Lactic Acid Bacteria (LAB) have found a very important role for their biotechnological applications in food industry. In general, LAB are considered as safe and food grade microorganisms because they do not cause any health risks to their host and many of them are recognized as probiotics [1]. However, there are reports that certain strains of LAB are harmful in immune-compromised individuals [2]. Among LAB, certain strains of *Weissella confusa* (basonym: *Lactobacillus confusus*) [3] were found to be an opportunistic pathogen in immune-compromised man causing endocarditis [4], bacteraemia [5], in foal causing septicaemia [6], infection in rainbow trout [7] and in primates [8] as well. However, a number of studies have also shown them as potential probiotic microorganisms [1, 5, 9, 10] and known to have antifungal [11] and antibacterial activity [5]. *W. confusa* have been isolated from a number of food sources [5] and known to produce various products such as lactic acid, bacteriocins, exopolysaccharides [5, 12]. Bacteriocins are known to be produced by different species of *Weissella* such as weissellicin L [13], weissellicin D [14], weissellicin Y and weissellicin M [15] by *W. hellenica*; weissellin A [16] by *W. paramesenteroides* DX; weissellicin 110 [17] by *W. cibaria* 110. However, to our knowledge, there is a scarcity of information about bacteriocins or antibacterial peptides (ABPs) produced by *W. confusa*.

The aim of this study was to purify, characterize, and evaluate ABP produced by *W. confusa* AJ79, a non-pathogenic isolate [18] from traditional Indian food source, uttapam batter fermented with *Piper betle* L. leaves.

II. Materials and Methods

Microorganisms and chemicals

The indicator strains *Staphylococcus aureus* subsp. *aureus* MTCC737, *Listeria monocytogenes* MTCC657, *Aeromonas hydrophila* subsp. *hydrophila* MTCC1739, *Escherichia coli* MTCC728, *Bacillus cereus* MTCC1272, *Bacillus licheniformis* MTCC429, *Bacillus subtilis* MTCC619, *Citrobacter freundii* MTCC1658, *Clostridium sporogenes* MTCC2684, *Clostridium perfringens* MTCC450, *Klebsiella pneumoniae* MTCC3384, *Mycobacterium smegmatis* MTCC6, *Micrococcus luteus* MTCC106, *Proteus vulgaris* MTCC426, *Pseudomonas aeroginosa* MTCC2295, *Staphylococcus epidermidis* MTCC3382 and *Vibrio parahaemolyticus* MTCC451 were procured from Microbial Type Culture Collections (MTCC), Institute of Microbial Technology, Chandigarh,

India. The microbial culture media, analytical grade chemicals were procured from HiMedia, India and Merck, India, and enzymes from Sigma-Aldrich, USA. The isolate *W. confusa* AJ79 (JX683269) [18] was used for the production and characterization of ABPs.

Purification of bacteriocin

The antimicrobial activity of ABP produced by *W. confusa* AJ79 was checked at each purification step against *L. monocytogenes* MTCC657 and *A. hydrophila* subsp. *hydrophila* MTCC1739 by agar well diffusion assay [19]. The antimicrobial activity was expressed as arbitrary units per millilitre (AU/mL) using formula $a^b \times 100$, where 'a' is dilution factor and 'b' the last dilution that produced an inhibition zone of at least 10 mm in diameter (including 6.0 mm of well diameter) [20], while the specific activity of the purified bacteriocin was calculated on the basis of activity units and protein content in the sample. The concentration of protein in the active fractions was estimated using Lowry's protocol [21].

SP-Sepharose Fast Flow Cation Exchange Chromatography

The isolate *W. confusa* AJ79 was grown in 1.0 L of de Man Rogosa Sharpe (MRS) broth culture media (pH 6.8) at 35°C for 48 h and the cell free supernatant (CFS) of culture was collected by harvesting the cells (centrifugation at 10000 *g*, 10 min). The CFS (pH 4.7-4.9) was directly loaded onto a 10.0 cm long (internal diameter 1.5 cm) SP-Sepharose fast flow cation exchange column calibrated with the distilled water. The active fraction was eluted with the 1.0 mol/L NaCl solution (pH 6.9) in distilled water.

C18 Sep-pak cartridges hydrophobic chromatography

The active fractions eluted from SP-Sepharose fast flow cation exchange column was passed through hydrophobic C18 Sep-pak cartridge which was activated with 50% of acetonitrile (MeCN) containing 0.1% trifluoroacetic acid (TFA) and calibrated with water. The active fractions were eluted with the 5, 10 and 40% of MeCN containing 0.1% TFA.

Sephadex G-25 (superfine) Gel permeation chromatography

The most active fraction(eluted with 40% of MeCN)from C18 Sep-pak cartridges was passed through Sephadex G25 superfine grade gel permeation column (50 cm × 1 cm) for the polishing of fractions. The fractions were eluted at the flow rate of 0.260 mL/min and detected at 280 nm using UV-visible spectrophotometer (Shimadzu, Japan). The active fractions were re-passed through this column to obtain a Gaussian peak. The active fractions in the Gaussian peak were pooled and concentrated *in vacuo* using rotavapor (BUCHI, Flawil, Switzerland). This ABP (bacteriocin) was designated as 'BAC79'; 'BAC' for bacteriocin and '79' for the isolate AJ79.

Determination of molecular weight of BAC79 by SDS-PAGE

The MW of the purified BAC79 was determined by Tris-tricine SDS-PAGE [22] using a vertical slab gel apparatus with a stacking gel of 6.5%, a spacer gel of 10.5%, and a separating gel of 16.5% acrylamide-bisacrylamide. The purified bacteriocin, BAC79 and molecular weight markers (Sigma) were run at 50 V for 12 h. After electrophoresis, the gel was cut into two halves. The one-half of the gel was fixed with 5% of glutaraldehyde and stained with Coomassie brilliant blue G 250 dye. The molecular weight was determined by comparing with the molecular weight marker (Sigma, Banglore) using GeneTools software of gel documentation system (Syngene, Cambridge). The other half of the gel was fixed with 20% isopropanol-10% acetic acid, washed with double distilled water for 4h and overlaid with 10^6 CFU/mL of *L. monocytogenes* MTCC657 in tryptic soya broth (TSB) having 1% (w/v) agar for bioassay [23].

MALDI-TOF-MS/MS analysis of bacteriocin BAC79

The active BAC79 band in the 1D Tricine SDS-PAGE gel was sliced gently, destained, eluted and treated with the trypsin enzyme. The cleaved peptide fragments were analyzed using MALDI-TOF spectrometer (Bruker Daltonics, Bremen, Germany) and the peptide mass fingerprint (PMF) data were recorded. The PMF peak data were searched at MASCOT server (http://www.matrixscience.com/). The search parameters at MASCOT server were set as follows: database: NCBInr; enzyme: trypsin, cuts C-term side of K and R unless next residue is P; fixed modifications: carbamidomethyl (C); variable modifications: oxidation (M); the peptide tolerance: 2 Da; mass values: monoisotopic; peptide charge state: 1+; max missed cleavages: 1. The selected peptide peaks in the PMF were analyzed by MS/MS. The obtained MS/MS peptide fingerprint was analyzed for the de novo amino acid sequencing.

Antimicrobial spectrum of purified bacteriocin BAC79

The antimicrobial activity of purified BAC79 was evaluated by agar well diffusion assay [19] against different gram-positive and gram-negative microorganisms. The Sephadex G-25 SF purified fraction of bacteriocin BAC79 in 5.0 mM acetate buffer (pH 5.5) was used for the antimicrobial assay. Briefly, the indicator microorganism was seeded in an appropriate agar containing culture medium by pour plate method. The well was created using sterile well borer. The well was sealed with 10 µL of 1% molten soft agar followed by instilling the well with 20µL of bacteriocin BAC79 (800 AU/mL).

Effect of enzymes, detergents, temperature and pH on bacteriocin activity

The bacteriocin BAC79 (800 AU/mL) was treated with enzymes at the final concentration of 1 mg/mL of trypsin, pepsin, proteinase-K, carboxypeptidase, lipase, and α-amylase for 2 h and evaluated for its antimicrobial activity against *L. monocytogenes* MTCC 657. The bacteriocin sample without enzyme treatment processed in a similar way was used as control [24].

Effect of pH on bacteriocin activity

The bacteriocin samples (800 AU/mL) were adjusted to pH 2 to 11 using either 2.0M HCl or 2.0M NaOH and incubated for 2 h. After incubation, the pH of samples was adjusted to 6.0 and its activity was checked against *L. monocytogenes* MTCC657 (10^8 – 10^9 cfu/mL) using agar well diffusion method [24].

Effect of temperature on bacteriocin activity

The bacteriocin BAC79 samples (800 AU/mL) were incubated at temperatures 25, 35, 45, 70, 90, and 100 °C for 1 h and 121°C for 15 min (autoclaving). Then the remnant activity was checked using agar well diffusion method against *L. monocytogenes* MTCC657 (10^8 – 10^9 cfu/mL) [24].

Effect of detergents on bacteriocin activity

The ABP (800 AU/mL) samples were incubated for 2 h at 37°C after treating individually with the final concentration of 1% (w/v) of either Triton X-100 or sodium dodecyl sulphate (SDS) or Tween 80 or Tween 20 or Urea or NaCl, 10 mmol/L ethylenediamine tetraacetic acid (EDTA), 0.1% β-mercaptoethanol (β-ME) and 0.1 % of dithiothreitol (DTT). The remnant activity was checked against *L. monocytogenes* MTCC657(10^8 – 10^9 cfu/mL) by agar well diffusion assay [24].

Effect of storage on bacteriocin activity

The ABP was stored at – 20 or 4 °C for 4 months and its antimicrobial activity was determined at 1, 2, 3 and 4 months against *L. monocytogenes* MTCC657 (10^8 – 10^9 cfu/mL) by agar well diffusion assay [24].

III. Mode of Action

Growth of pathogen in presence of bacteriocins

The growth of *L. monocytogenes* MTCC657 was checked in presence of BAC79 by inoculating the *Listeria* cells (10^6 cells/mL) in TSB broth and incubated at 37 °C for 2h. After incubation, the ABP (800 AU/ml) was added to the culture. The OD of the cells was recorded at 600 nm for every hour up to 12 h and the viable cells were counted after 5 and 12 h of BAC79 addition [25]. The *L. monocytogenes* MTCC657 in TSB without bacteriocin treatment was used as a control.

Morphological changes in the membrane of microbe pathogen by bacteriocin BAC79

The *L. monocytogenes* MTCC657 was grown in the TSB broth for 15-18 h. The cells were harvested by centrifugation (8000 *g*, 10 min), washed with and suspended in PBS of its original volume. The ABP BAC79 (800 AU/mL) was added to the cell suspension and incubated for 2 h at room temperature followed by harvesting of cells (8000 *g*, 10 min). The collected cells were washed with PBS, fixed with 2% formaldehyde for 4 h followed by postfixing with 1% of OsO_4 (Sigma, Bangalore). The cells were dehydrated with the series of gradient ethanol (10-100%) and dried *in vacuo* using speed vacuum concentrator(Christ, Osterode, Germany) [24]. The dried cells were scanned under scanning electron microscope (SEM) (S-3400N; Hitachi Asia Ltd., Tampines Grande, Singapore).

Release of UV-Absorbing materials from pathogen by bacteriocin BAC79

The *L. monocytogenes* MTCC657 was grown in the TSB broth for 15-18 h. The cells were harvested by centrifugation (8000 *g*, 10 min), washed with and suspended in PBS of its original volume. The AMP (800 AU/mL) was added to the cell suspension and incubated for 2 h at room temperature. The CFS was collected after incubation by harvesting of cells (8000 *g*, 10 min) and filtered using 0.22 µ syringe filter. The absorbance was measured at 220, 250 and 280 nm [26]. The cells without bacteriocin served as control.

IV. Results and Discussion

The present study deals with the purification and characterization of the antimicrobial peptides (bacteriocins) produced by the potent isolate, *W. confusa* AJ79 from uttapam batter fermented supplementing with *Piper betle* L. leaves. The bacteriocins in the CFS of *W. confusa* AJ79 were captured onto cation exchange SP-Sepharose column and eluted with 1.0 M of NaCl solution indicating the bacteriocins were cationic in nature. Subsequently, ABPs were purified using hydrophobic interaction by passing through the C18 cartridge (Waters Classic C18) and eluting with 5, 10 and 40% of MeCN containing 0.1% TFA. All the three fractions (5, 10 and 40% MeCN) exhibited activity against *L. monocytogenes* MTCC657 with inhibition zones of 13, 10 and 16 mm respectively. This indicated that the isolate *W. confusa* AJ79 is a producer of multiple bacteriocins, i.e., three cationic bacteriocins having varied hydrophobic characteristics. There are reports that have shown different microorganisms producing multiple bacteriocins [27-29]. The bacteriocin fraction eluted with 40% MeCN showing higher activity was designated as BAC79 and the same was purified and characterized further.

Purification of bacteriocin BAC79

The activity, yield and fold purification after various purification techniques are summarized in Table 1. Purification step with a C18 cartridge for BAC79 resulted in the yield of 20% having a specific activity of 32.65 AU/mg and the purification fold was 14.38 (Table 1). When it was further purified with the G25SF Sephadex column, the yield was 16%, specific activity 258.06 AU/mg with the 113.68 fold purification. The Gaussian peak obtained in GPC indicates the homogeneous nature of the sample (Fig. 1). The production of potent bacteriocin BAC79 can be further enhanced by optimizing the media components.

SDS-PAGE analysis

The tricine SDS-PAGE analysis shown that the molecular weight of the BAC79 bacteriocin was about 5 kDa (Fig. 2a) which was within the mass range of the most of the bacteriocins from the genus *Weissella* [13, 15-17]. The *in situ* antimicrobial activity of the peptide in PAGE gel against *L. monocytogenes* MTCC 657 was confirmed by agar gel overlay method (Fig. 2b).

Trypsin digestion and sequence analysis

After eluting the active band from the gel it was digested with the trypsin enzyme. The resulting peptide fragments were analyzed using MALDI-TOF-MS. The MASCOT server (http://www.matrixscience.com) was used to analyse the mass data to identify the peptide sequence by matching the peptide mass fingerprint of known peptides. A total of 9 peptide fragments with different *m/z* ratios (804.34, 806.35, 842.56, 861.09, 877.07, 1278.70, 1487.86, 1941.08 and 2211.27) of BAC79 were obtained after trypsin hydrolysis. The complete identification of all the enzymatic fragments could not be done at MASCOT server indicating BAC79 was an unknown peptide.

During MASCOT search, the peptides with *m/z* 804.34, 842.56, 861.09, 1278.70, 1941.08 and 2211.27 were matched with the protein gi|443488077 (D-alanyl-D-alanine carboxypeptidase) with top score of 81 which was not significant ($p>0.05$); whereas the peptides *m/z* 806.35, 877.07 & 1487.86 were not matched.

The MS/MS analysis was performed for the peptide fragments of *m/z* 842.56, 1278.70 and 2211.27. The de novo amino acid sequencing was performed using MS/MS ion data. The amino acid sequence was deduced as 'AIAVELAR' (for *m/z* 842.56), 'DVFEAIQTLSR' (for m/z 1278.70) and 'GSVFQDMPLVSNKELMDLR' (for *m/z* 2211.27). However, it had no homology with any known bacteriocin or antimicrobial peptides. The bacteriocin BAC79 was considered an unknown protein molecule as its peptide mass fingerprint when analyzed using MASCOT server (http://www.matrixscience.com) had no homology with any known peptide or protein and this may be due to the scarcity of information at database particularly about proteins or peptides from *W. confusa*. Moreover, the amino acid sequences of peptide fragments (*m/z* 842.56, 1278.70 and *m/z* 2211.27) had no similarity with any known antimicrobial peptides or bacteriocins.

Antibacterial spectrum

The bacteriocin BAC79 exhibited a wide spectrum of activity against different gram-positive and gram-negative microorganisms (Table 2). Conversely, the bacteriocins reported from the other *Weissella* species [13, 15-17] had a narrow spectrum of antimicrobial activity, especially against gram-positive microbes. The BAC79 was very much active against *L. monocytogenes* evident from agar gel overlay method (Fig. 2b).

Effects of enzymes, detergents, temperature and pH on bacteriocin BAC79

The antimicrobial activity of BAC79 was lost completely after treatment with enzymes trypsin, pepsin, proteinase-K, and carboxypeptidase while there was no loss of activity with either amylase or lipase. The BAC79 was stable to different detergents such as triton X-100, tween 80, SDS and denaturants DTT, EDTA and β-ME (Table 3).The BAC79 was stable up to 100°C and when exposed to 121°C, a minimal reduction in the

activity was observed reflecting its stability during autoclaving. The storage at a lower temperature (4°C) had no effect on its activity at least for 4 months (Table 4). The activity of BAC79 was not affected in the pH range of 2-6.

Mode of action on target cells

The growth of the *L. monocytogenes* MTCC657 was reduced (43%) after bacteriocin (BAC79) treatment within 2 h, while there were no observable colonies after 12 h, compared to luxurious growth observed in the case of control untreated *Listeria* (Table 5). It was a membrane active peptide which had inhibited the growth of *Listeria* by disrupting its cell membrane inferred from the morphological changes in the membrane under SEM examination (Fig. 3) and spectrophotometric detection of the release of UV absorbing materials in its milieu (Table 6). The membrane disruption might be a cause for the release of UV absorbing materials (cellular components basically proteins, nucleic acids) out of the *Listeria* cells when exposed to the BAC79 leading to the death of the microbe. Like other bacteriocins, it was degraded by proteolytic enzymes such as trypsin, pepsin, proteinase-K and carboxypeptidase. Based on its characteristics the bacteriocin BAC79 can be brought under class II of bacteriocins and considered useful for pharmaceuticals and food applications.

V. Conclusion

In conclusion, in this study, a bacteriocin (BAC79) was purified and characterized from an isolate of *W. confusa* AJ79. The BAC79 was an unknown bacteriocin of approximate MW 5 kDa and exhibited a broad range of inhibitory activity against different foodborne pathogenic microorganisms. The bacteriocin BAC79 has strong thermal and pH stability that enables its possibility of future food biotechnological applications.

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Conflict Of Interest

The authors declare that they have no conflict of interest.

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Table 1: Purification and activity of bacteriocin BAC79 produced by *W. confusa* AJ79 against *Listeria monocytogenes* MTCC 657

Fraction	**Volume (mL)**	**Protein concentration mg/mL**	**Activity AU/mL**	**Total Activity (U)**	**Specific Activity (AU/mg)**	**Purification Fold**	**Yield (%)**
CFS	100	88.0	200	20000	2.27	1.00	100
SP Sepharose	20	65.5	400	8000	6.11	2.69	40
C-18 Sep-pak	5	24.5	800	4000	32.65	14.38	20
Sephadex G25 SF	2	6.2	1600	3200	258.06	113.68	16

Table 2: Antimicrobial activity of bacteriocin BAC79 produced by *W. confusa* AJ79

Indicator Strain	Zone of inhibition*	
	BAC79	Ampicillin (10 micrograms)
Aeromonas hydrophila subsp. *hydrophila* MTCC1739	20	23
Bacillus cereus MTCC1272	16	27
Bacillus licheniformis MTCC429	21	25
Bacillus subtilis MTCC619	16	22
Citrobacter freundii MTCC1658	16	24
Clostridium sporogenes MTCC2684	17	24
Clostridium perfringens MTCC450	15	25
Escherichia coli MTCC728	20	26
Klebsiella pneumoniae MTCC3384	19	32
Listeria monocytogenes MTCC657	18	33
Mycobacterium smegmatis MTCC6	17	25
Micrococcus luteus MTCC106	18	31
Proteus vulgaris MTCC426	19	26
Pseudomonas aeroginosa MTCC2295	22	28
Staphylococcus aureus subsp. *aureus* MTCC737	20	32
Staphylococcus epidermis MTCC3382	17	29
Vibrio parahaemolyticus MTCC451	17	24

*Values are mean of three independent experiments performed in duplicates expressed in millimetres inclusive of well diameter 6 mm.

Table 3: Effect of enzymes and detergents/denaturants on bacteriocin BAC79 activity

	Activity* (mm)
Control	24
Enzymes	
Proteinase K	0
Pepsin	0
Trypsin	0
Carboxypeptidase	0
Lipase	22
α-amylase	22
Detergents/Denaturants	

Triton X-100 (1%)	23
Tween 80 (1%)	23
SDS (1%)	23
NaCl (1%)	24
EDTA (10 mM)	24
β-ME (0.1%)	24
DTT (0.1%)	23

*Values are mean of three independent experiments performed in duplicates expressed in millimetres inclusive of well diameter 6 mm.

Table 4: Effect of pH, temperature and storage on bacteriocin BAC79 activity

	Activity* (mm)
pH	
2	21
4	21
6	20
8	16
9	0
Temperature (°C)	
25	21
45	21
70	21
100	20
121	20
Storage (°C)	
-20°C, 2 months	22
4°C, 1month	22
4°C, 2 months	20
4°C, 4 months	20

*Values are mean of three independent experiments performed in duplicates expressed in millimetres inclusive of well diameter 6 mm.

Table 5: Effect of Bacteriocin BAC79 on growth of *Listeria*

	log CFU/mL		
	0h	5h	12h
Control *Listeria*	10.4± 0.04	11.2±0.13	13.5±0.09
Treated with BAC79	10.3±0.02	5.9±0.01	No colonies

Table 6: Release of UV absorbing materials from *Listeria* upon treatment with bacteriocin BAC79

	OD at 220 nm	OD at 250 nm	OD at 280 nm
Control *Listeria*	0.365±0.0035	0.382±0.0007	0.371±0.0091
Treated with BAC79	0.407±0.0007	0.401±0.0014	0.405±0.0035

Figure 1: Gaussian peak of active fractions obtained when re-passed to G-25 SF: the bar shows the active fractions pooled

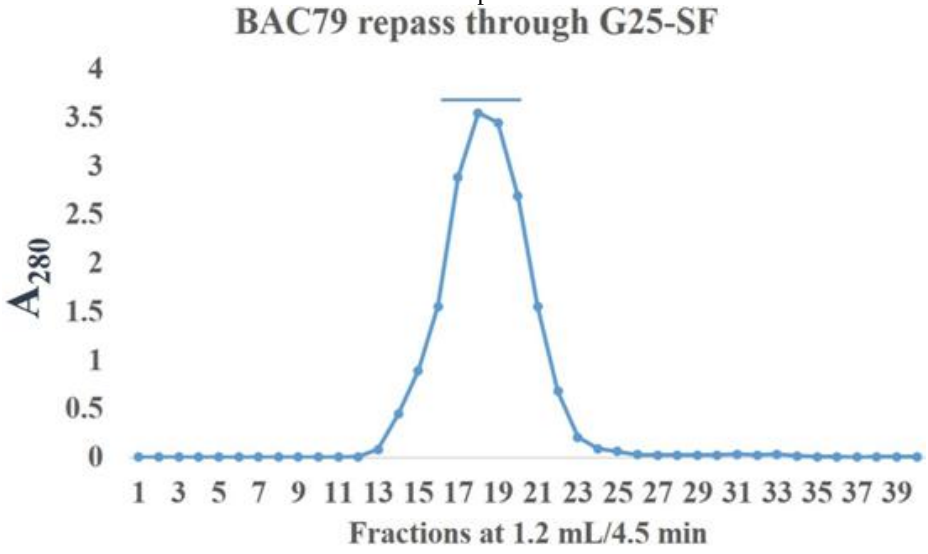


Figure 2: (a) Tricine SDS-PAGE electrogram showing the bacteriocin BAC79 (approximate molecular weight 5 kDa) and **(b)** In situ antimicrobial activity of BAC79 in Tricine PAGE gel against *Listeria monocytogenes* MTCC657 using agar gel over lay method

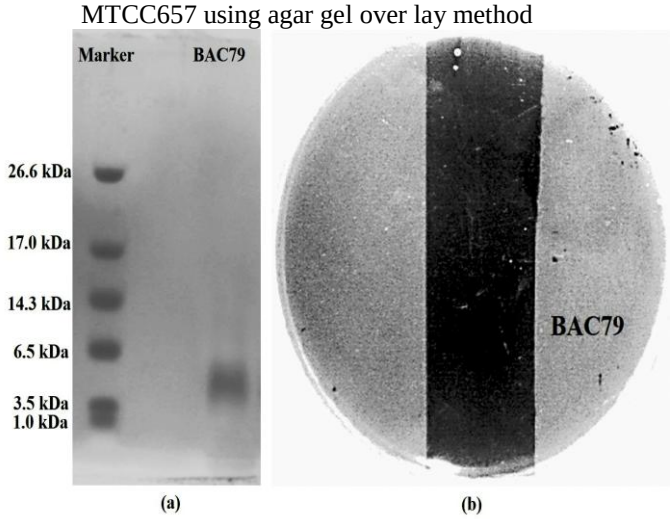
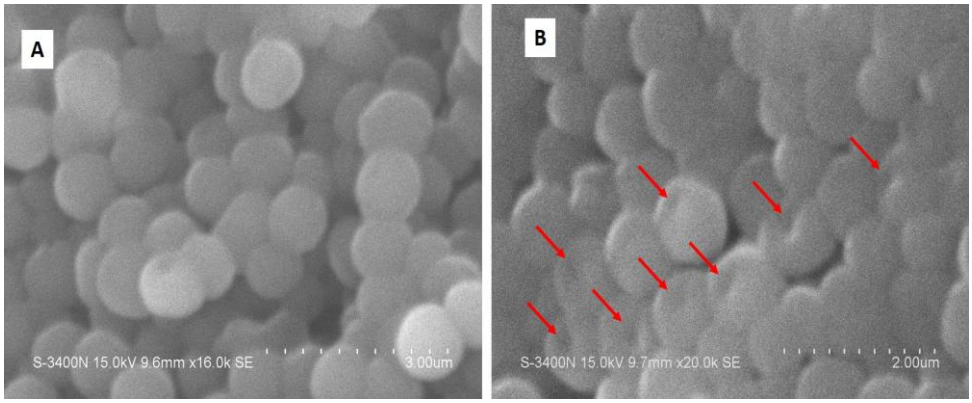


Figure 3: Scanning electron micrograph showing pore formation on the cell membrane of senstive organism: **(A)** Control untreated *L. monocytogenes* MTCC657 and **(B)** *L. monocytogenes* MTCC657 cells treated with bacteriocin BAC79



Ashwini Kumar Dubey "Purification and Characterization of an Antibacterial Peptide (Bacteriocin) Produced By *Weissella confusa* AJ79."." IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 13.2 (2018): 17-24.