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Characterization, Cytotoxic Analysis and Action Mechanism of Antilisterial Bacteriocin Produced by *Lactobacillus plantarum* Isolated from Cheddar Cheese

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Abstract
Emergence of food borne pathogens and multidrug resistant organisms has enforced the screening of natural antimicrobial compounds. Bacteriocins are ribosomally synthesized natural antimicrobial peptides produced by numerous bacteria. These antimicrobial peptides are gaining more attention as alternative therapeutics in pharmaceutical industry as next-generation antibiotics targeting the multi-drug resistant pathogens and as a bio preservative in food industry. The current study is aimed to purify and characterize a broad spectrum bacteriocin (Bac-IB45) with the determination of its plausible mode/mechanism of action. In this research, Bac-IB45 from *Lactobacillus plantarum* KIBGE-IB45 was 41.71-fold purified with the molecular mass of 20.5 kDa estimated using tricine SDS-PAGE. This bacteriocin is highly thermostable and pH stable in nature. It also exhibited stability against various metal ions, surfactants and organic solvents. Purified Bac-IB45 showed broad antimicrobial potential against various multidrug resistant, food borne bacterial and fungal pathogens. However, Bac-IB45 was found to be sensitive to various proteolytic enzymes. The time-kill assay of Bac-IB45 and scanning electron microscopic analysis revealed the bactericidal mode/mechanism of action against *Listeria monocytogenes* ATCC 7644. This unique Bac-IB45 with broad spectrum of inhibition and bactericidal mode of action could be used as a natural food preservative and as alternative therapeutics to solve the unrestrained problems of industries.

Keywords Gel permeation chromatography · *Listeria monocytogenes* · Scanning electron microscope · Thermostable bacteriocin · Tricine SDS-PAGE

Introduction

In recent years, deterioration and spoilage of food remains a debated issue despite modern advances in food science and technology. The emergence of food borne pathogens poses a serious health and quality concerns. Among various food borne pathogens, *Listeria monocytogenes* is the most common and formidable pathogen causing life-threatening disease which results in death. In the last few years, consumers are aware of the health hazards regarding the use of chemical preservatives in food products. Therefore, there is a clear need to establish some natural strategies to enhance the shelf-life and overcome the deterioration of food products. The preservation of foods by natural antimicrobial compounds may be a satisfactory approach to reduce the incidence of food borne illnesses. Among various antimicrobial compounds, antimicrobial Peptides (AMPs) have gained great interest to resolve these critical issues and are being continuously explored for their

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potential antimicrobial applications (Strempel et al. 2015; Kang et al. 2017).

Bacteriocins are one of the AMP produced by bacteria and recognized as ribosomally synthesized natural bioactive peptides. They have several properties that make them suitable as a natural food preservative. Generally, bacteriocins have exhibited broad antimicrobial spectrum with bactericidal mode of action against various bacterial and fungal strains. One of the main properties is the inactivation of bacteriocin by digestive proteases. In food industry, bacteriocins from lactic acid bacteria (LAB) have gained interest because of the generally regarded as safe (GRAS) status (Castellano et al. 2008; Wilson et al. 2011; Maurya and Thakur 2012). In recent years, research on LAB bacteriocins has received great attention due to its dual use as a natural food preservative and alternative therapeutics (Cotter et al. 2013). However, there is still a need to learn more about LAB bacteriocins due to the increasing number of new bacteriocins in this group with unique properties.

Antimicrobial peptides produced by different organisms exhibit either membrane disruptive mechanisms and/or non-membrane disruptive mechanism which inhibit the intracellular components (Giuliani et al. 2007). Generally, bacteriocins disrupt the cell membrane by its amphipathic structure. Due to the variations in the chemical structure, bacteriocins affect different essential functions of a cell. The most common mechanism of bacteriocins is the formation of pores into the cell membrane which causes leakage of cellular components (Parapouli et al. 2013). Nisin is the only bacteriocin whose mode of action has been well studied. It is the only FDA (Food and Drug Administration) approved bacteriocin produced by *Lactococcus lactis* used for the preservation of food. However, the practical application of nisin is limited because of its low antibacterial activity against Gram negative bacteria and in foods with alkaline pH. Therefore, the exploration of new bacteriocins is in full swing due to the limitations of commercially available bacteriocins and it became a challenge for researchers to investigate broad spectrum bacteriocins having stability at wide range of pH and temperatures for longer period.

Keeping all these considerations in view, this study is aimed to purify and characterize a new bacteriocin from LAB with broad antimicrobial potential against food borne pathogens to increase the shelf life of the food products. Furthermore, the action mechanism and cytotoxic effect of purified bacteriocin were also evaluated for its future applications.

Materials and Methods

Microorganisms and Culture Conditions

Lactobacillus plantarum KIBGE-IB45 [GenBank Accession number: MG814034] was previously isolated from cheddar cheese and cultivated in MRS (DeMan Rogosa Sharpe, Oxoid) medium at 35 °C for 24 h to produce bacteriocin (Ibrahim et al. 2019). Moreover, *Listeria monocytogenes* ATCC 7644 was selected as an indicator strain and grown in nutrient agar for 24 h at 37 °C. Whereas, other bacterial and fungal strains were used to check the antimicrobial spectrum of bacteriocin from *L. plantarum* KIBGE-IB45. All the bacterial strains were cultivated for 24 h at 37 °C and fungal strains at 25 °C for 5 days. For long term storage, all the cultures were stored in 15% glycerol at − 20 °C.

Bacteriocin Production

To produce bacteriocin, overnight culture of *L. plantarum* KIBGE-IB45 (10^8 CFU mL^{-1}) was inoculated in MRS medium and incubated for 24 h at 35 °C. After production, the medium was centrifuged at 3000×*g* at 4 °C for 15 min and the extracellular bacteriocin was obtained in the cell free supernatant (CFS). The CFS was filter sterilized using 0.22 µm filter (Millipore, USA). To eliminate the effect of organic acids, CFS was adjusted to pH 6.0 through sterilized NaOH (5 N). Moreover, the inhibitory effect of hydrogen peroxide (H_2O_2) was also eliminated by the treatment of neutralized CFS with 1 mg mL^{-1} catalase for 30 min at 25 °C. Agar well diffusion assay was used to determine the antibacterial activity of CFS. For this purpose, *L. monocytogenes* ATCC 7644 (10^6 CFU mL^{-1}) was spread on pre incubated nutrient agar plates. 100 µL of CFS was added into the wells (5 mm) and the nutrient agar plates were incubated at 37 °C for 24 h (Ansari et al. 2015).

Growth Curve of *Lactobacillus plantarum* KIBGE-IB45

Growth kinetics of *L. plantarum* KIBGE-IB45 was examined along with bacteriocin production at various stages of microbial growth cycle. For this purpose, *L. plantarum* KIBGE-IB45 (10^8 CFU mL^{-1}) was inoculated in MRS medium and incubated at 35 °C for 24 h. During incubation, samples were taken after different time intervals till 48 h and the cell density was measured at 600 nm. Production of bacteriocin was also checked at different stages of *L. plantarum* growth cycle using agar well diffusion method against *L. monocytogenes*.

Purification of Bacteriocin

The collected cell free supernatant (CFS) was purified using different steps. Initially, the CFS was subjected to partial purification through ammonium sulphate (20–80%) fraction precipitation. In this, ammonium sulfate was gradually added into the CFS till 80% saturation was achieved and kept at stirring for 20 h at 4 °C. After 20 h, the precipitates were collected and dissolved in sodium acetate buffer (50 mM pH 6.0). The precipitates were dialyzed through a dialysis tube (2.0 kDa cut off, Sigma) and sterilized with membrane filter (Millipore, USA) of 0.22 µm. After dialysis, the bacteriocin was purified using gel permeation chromatography. Briefly, the dialyzed sample (3.0 mL) was loaded on Sepharose CL-6B column (GE Healthcare, Sweden), pre-equilibrated with 50 mM sodium acetate buffer (pH 6.0). All the fractions were collected with the flow rate of 0.5 mL min^{-1} and total protein concentration was measured at 280 nm. In addition, all the fractions were examined for antibacterial activity using agar well diffusion method and the fractions that exhibited predominant antibacterial activity against *L. monocytogenes* were combined. The pooled sample was then subjected to centricon centrifugal filter device (Ultracel YM-10) of cut off 10 kDa (Millipore, USA) to concentrate the sample. After each step Lowry's method was used to determine the total protein concentration of samples using bovine serum albumin as standard (Lowry et al. 1951). The purified bacteriocin was lyophilized and designated as Bac-IB45 for further studies.

Determination of Arbitrary Unit and Minimal Inhibitory Concentration of Bac-IB45

In order to calculate the arbitrary units (AU), two-fold serial dilutions of purified Bac-IB45 were prepared in 50 mM sodium acetate buffer (pH 6.0). AU is defined as the reciprocal of the highest dilution of antimicrobial compound which shows an inhibition of the tested strain. Nutrient agar plates were spread with *L. monocytogenes* and the dilutions of Bac-IB45 (100 µL) were added into the prepared wells and plates were incubated at 37 °C for 24 h. After incubation, inhibitory zones in millimeters were measured and the antibacterial potential of Bac-IB45 was expressed in AU mL^{-1} (Ansari et al. 2018).

To measure the minimal inhibitory concentration (MIC) of Bac-IB45, tube dilution method was used. In this method, purified Bac-IB45 was two-fold serially diluted with an initial concentration of 0.65 mg mL^{-1}. Dilutions were prepared in sodium acetate buffer (50 mM, pH 6.0) and 100 µL of *L. monocytogenes* (10^6 CFU mL^{-1}) was added in each dilution tube. All the tubes were incubated for 24 h at 37 °C. Afterwards, the visible growth of the *L. monocytogenes* was examined in each tube and to further confirm the inhibition of the *L. monocytogenes,* plate count method was used. The highest dilution with no bacterial growth is measured as the minimal inhibitory concentration of Bac-IB45.

Characterization of Bac-IB45

Molecular Weight Estimation

The molecular mass of Bac-IB45 was estimated using Tricine SDS-PAGE and the electrophoretic mobility of purified bacteriocin was compared with the mobility of low range protein marker of multicolor spectra (MW 1700–42,000 Da, Thermo Scientific). Electrophoresis was performed using a method describe by Schägger (2006) with slight modifications. Mini-PROTEAN® TGX™ precast gels of BIORAD (4–20%) were used for the better separation of low molecular weight proteins. After electrophoresis, one of the gels was stained with Coomassie brilliant blue (R-250) for molecular weight estimation and another gel was left unstained. To confirm the antibacterial potential of the band of interest, the unstained gel was rinsed several times with sterilized Milli-Q® water and overlaid using nutrient agar (1%) containing log phase culture of *L. monocytogenes* (10^6 CFU mL^{-1}). The nutrient agar plate was incubated for 24 h at 37 °C to examine the zone of inhibition.

Thermal and pH Stability of Bac-IB45

The stability of Bac-IB45 was determined after treatment with different physical and chemical parameters for its applications in food and pharmaceutical industries. For the evaluation of thermal stability, the influence of various temperatures ranging from 50 to 100 °C on Bac-IB45 was determined. Samples were drawn at different time intervals up to 4 h. Moreover, in order to determine the pH stability, Bac-IB45 was treated with different pH buffers. For this, four different buffers were used including citrate buffer (50 mM, pH 3–5), sodium acetate buffer (50 mM, pH 5.5–6.5), phosphate buffer (50 mM, pH 6.5–7.5) and glycine NaOH (50 mM, pH 8–9). Purified Bac-IB45 was incubated in the buffer solution for 2 h at 4 °C. Buffers without Bac-IB45 were used as a control. After treatments, the percent residual activity was determined using agar well diffusion assay against *L. monocytogenes* and the plates were incubated at 37 °C for 24 h.

Effect of Metal Ions, Organic Solvents and Surfactants

Bac-IB45 was pre-incubated with various metal ions for 1 h at 4 °C. For this purpose, different metal ions of chloride salts were used in different concentrations of 1 mM, 5 mM

and 10 mM. Metal ions alone were used as a control. To examine the influence of organic solvents and surfactants on Bac-IB45, two different concentrations (20 mM and 50 mM) were used. All the organic solvents and surfactants alone were used as a control. After treatments of Bac-IB45 with metal ions, organic solvents and surfactants, the percent relative activity against *L. monocytogenes* was calculated using agar well diffusion assay.

Sensitivity to Proteolytic Enzymes

Effect of various proteolytic enzymes on Bac-IB45 was also determined. Bac-IB45 was pre-incubated with different enzymes including protease, pepsin, lysozyme (Serva, Germany) and Proteinase-K (Invitrogen) at 37 °C for 2 h at a concentration of 1 mg mL^{-1}. All the enzymes alone were used as a control. After treatment with enzymes, agar well diffusion assay was used to determine the percent relative activity of Bac-IB45.

Storage Stability of Bac-IB45

To determine the storage stability, Bac-IB45 was kept at various temperatures including: 37 °C, 4 °C, − 4 °C, − 10 °C, − 20 °C and − 80 °C for 6 months. For this purpose, samples were drawn and assayed initially on daily basis followed by months. Antibacterial activity was determined up to 6 months using agar well diffusion assay and the data were expressed in terms of percent residual activity.

Disk Diffusion Assay

Disk diffusion assay was performed to determine the antibiotic susceptibility pattern of multidrug resistant (MDR) bacteria, before evaluating the spectrum of Bac-IB45 against MDR strains. For this purpose, various antibiotics including Imipenem (10 µg), Doripenem (10 µg), Cefepime (30 µg), Vancomycin (30 µg), Ampicillin (10 µg), Ciprofloxacin (5 µg), Oxacillin (1 µg), Clindamycin (2 µg), Tobramycin (10 µg), Ertapenem (10 µg) were purchased from the local vendor (Oxoid Ltd., Hampshire, UK). In this method, log phase (10^6 CFU mL^{-1}) culture of each multidrug resistant bacteria was spread on nutrient agar plates and the antibiotic disks were placed on the agar surface. The plates were incubated at 37 °C for 24 h and susceptibility pattern was determined.

Inhibitory Spectrum of Bac-IB45

Inhibitory potential of purified Bac-IB45 was investigated using agar well diffusion method. For this purpose, numerous food borne and multidrug resistant bacteria (*Listeria monocytogenes, Pseudomonas aeruginosa,* Methicillin resistant *staphylococcus aureus, Bacillus cereus*, *Escherichia coli Enterococcus faecalis* and *Salmonella typhimurium*) and various fungal species of genera *Aspergillus* (*A. terreus*, *A. fumigatus*, *A. niger*, and *A. flavus*) were used. All the bacterial and fungal strains were cultured on nutrient agar (24 h at 37 °C) and potato dextrose agar (5 days at 25 °C), respectively.

Action Mechanism of Bac-IB45

In this study, two approaches were used for the determination of action mechanism of Bac-IB45.

Time-Kill Assay

To investigate the mode of action of Bac-IB45, time-kill assay was performed. In this assay, overnight culture of *L. monocytogenes* was grown to the log phase (10^6 CFU mL^{-1}) and inoculated in nutrient broth (100 mL). Afterwards, the minimal inhibitory concentration (80 µg mL^{-1}) of Bac-IB45 was also added in the same flask and incubated at 35 °C. The optical density was measured at 600 nm and the viable cell count was determined after every 02 h till 12 h using plate count method. To confirm the complete inhibition of *L. monocytogenes*, the regrowth of the organism was investigated after 24 h. *L. monocytogenes* without Bac-IB45 was used as a control.

Scanning Electron Microscopy

The ultrastructure changes in the *L. monocytogenes* was examined using scanning electron microscope (SEM). Briefly, log phase culture of *L. monocytogenes* was treated with 80 µg mL^{-1} of Bac-IB45 for 8 h. After treatment, cells were collected by centrifugation at 3000×*g* and washed thrice with 100 mM phosphate buffer saline (pH 7.4). The cells were fixed with glutaraldehyde (2.5%) for 1 h at 4 °C and washed again. Afterwards, the cells were dehydrated using gradient ethanol (30%, 50%, 70%, 90% and 100%) and dried under vacuum at 30 °C. The dried cells were imaged under scanning electron microscope (JSM-6380 A-Jeol, Japan).

In vitro Cytotoxic Analysis of Bac-IB45

The cytotoxic effect of Bac-IB45 was examined using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (Promega) assay. In this study, NIH/3T3 (primary mouse embryonic fibroblast cells) cell line was used for this purpose. The cells were maintained in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotics and 10% fetal bovine serum (FBS). NIH/3T3 cells (5×10^3/well) were plated in 96-well plates (in triplicate) and incubated with 5% CO_2 for 24 h at 37 °C. Cells were then treated with various concentrations of Bac-IB45 and again incubated at 37 °C with 5% CO_2 for 24 h, while 1% Triton X-100 and blank DMEM medium were taken as a positive and negative controls, respectively. After 24 h, MTS reagent (20 µL) was added in each well and plate was further incubated for 3 h. The plate was read at 490 nm in an ELISA plate reader (BIOBASE, China) and the cell viability was calculated after treated with Bac-IB45 in terms of percentage.

Statistical Analysis

All these experiments were conducted independently three times in triplicates and data are presented as mean ± standard deviation (SD). The difference between data sets were determined by Student's *t* test and the p value < 0.05 was considered as statistically significant.

Results and Discussion

Growth Kinetics Studies and Production of Bac-IB45

Production of Bac-IB45 was monitored at different stages of the growth cycle of the *Lactobacillus plantarum* KIBGE-IB45 and the results showed the time dependent production of Bac-IB45. It was observed that after 6 h of incubation *L. plantarum* was entered in the log phase and started bacteriocin production at 12 h. The maximum production was achieved at 24 h that is early stationary phase. The bacteria were in stationary phase from 24 to 36 h, after 36 h of incubation the growth of *L. plantarum* started to decline with the decrease in the antibacterial activity of Bac-IB45 (Fig. 1). This decrease might be due to the shift in the pH of the medium and because of the presence of proteases in the decline phase of bacteria. Production of Bac-IB45 in late logarithm or early stationary phase indicated that the bacteriocin from *L. plantarum* was a secondary metabolite. The total cell mass of *L. plantarum* was also higher at this stage. Similarly, Elayaraja et al. 2014 also reported the degradation of antibacterial activity of bacteriocin and the growth of *Lactobacillus* species after 36 h of incubation. In contrast, other studies reported the maximum production of bacteriocins produced by *L. plantarum* and *Bacillus subtilis* after 10 h and 18 h of incubation, respectively (Kormin et al. 2001; Ansari et al. 2015).

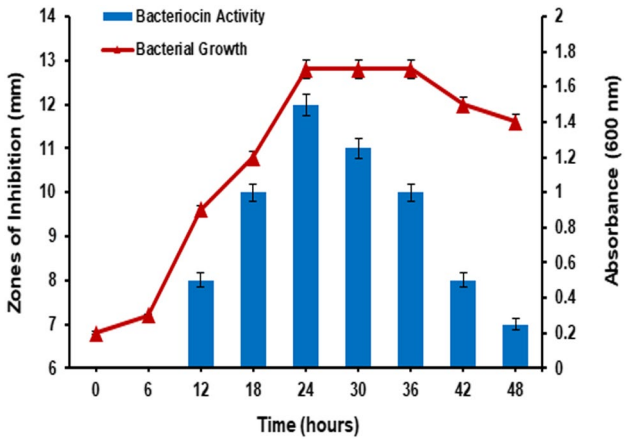

Fig. 1 Growth curve of *Lactobacillus plantarum* and antibacterial activity of bacteriocin (Bac-IB45) against *Listeria monocytogenes* ATCC 7644 in MRS medium at 35 °C for 48 h. The experiment was performed in triplicates and the error bars represent the standard deviation of the mean (p-value < 0.05)

Purification and Molecular Weight Estimation of Bac-IB45

After production, several steps were used for the purification of Bac-IB45. Initially, bacteriocin was precipitated out at 80% ammonium sulphate saturation. Desalting of Bac-IB45 was resulted in the gradual enhancement in the units and the specific activity of Bac-IB45 from 40 to 160 AU mL^{-1} and 2.95 to 17.73 AU mg^{-1}, respectively. Afterwards, gel permeation chromatography was used to purify Bac-IB45 and the fractions that showed strong antilisterial activity were pooled. It was noticed that Bac-IB45 was 8.70-fold purified with the specific activity of 25.68 AU mg^{-1}. The pooled fractions were further concentrated through centricon centrifugal filter device (cut off: 10 kDa) and the antibacterial activity was observed in the retainate with the rise in the fold purification and the specific activity from 8.70 to 41.71 times and 25.68 to 123.07 AU mg^{-1}, respectively. The data are presented in Table 1. The arbitrary units of the purified Bac-IB45 was 80 AU mL^{-1} (Fig. 2). Similarly, Sharma et al. (2011) also worked on complete purification of a bacteriocin from *B. subtilis* using a single step gel exclusion column chromatography with a final fold purification of 22.30 times.

Generally, bacteriocins possess a wide range of molecular weight hence, in this study Tricine SDS-PAGE with a gradient gel system was used for the estimation of molecular weight of Bac-IB45. Several studies reported the separation of low molecular weight bacteriocins by Tricine SDS PAGE includes paracin-1.7 of 11 kDa from *L. paracasei* HD1.7, entomocin 9 of 12.4 kDa produced

Table 1 Purification and antibacterial activity of Bac-IB45 produced by *Lactobacillus plantarum* KIBGE-IB45

Purification steps	Total volume (mL)	Arbitrary units (AU mL^{-1})	Total protein (mg mL^{-1})	Specific activity (AU mg^{-1})	Fold purification
Crude bacteriocin	990	40	13.55	2.95	1.00
Desalting	20	160	9.02	17.73	6.01
Sepharose CL-6B	5	160	6.23	25.68	8.70
Centricon (10 kDa)	2	80	0.65	123.07	41.71

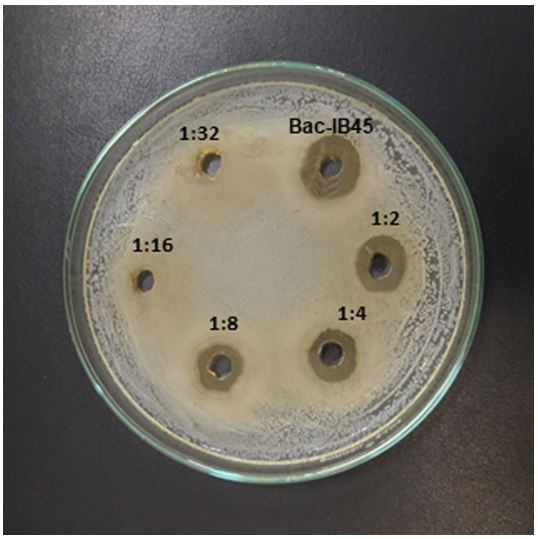

Fig. 2 Calculation of arbitrary units of purified Bac-IB45 against *Listeria monocytogenes* ATCC 7644 using agar well diffusion assay

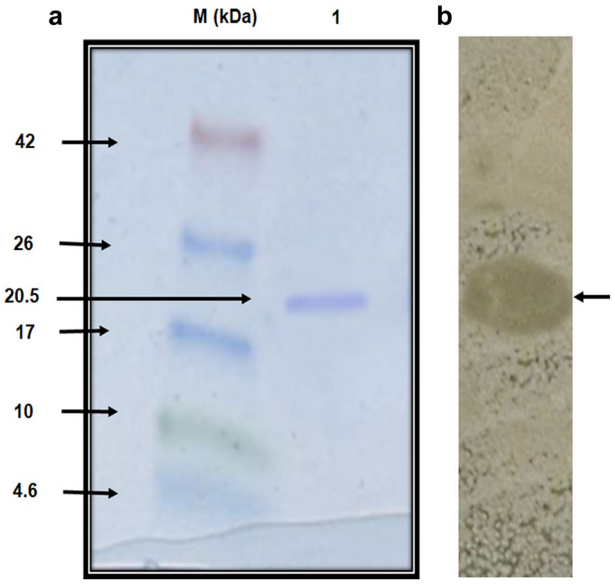

Fig. 3 **a** Tricine SDS-PAGE profile of Bac-IB45 produced by *Lactobacillus plantarum* KIBGE-IB45. Lane M: Low molecular weight marker (1700–42,000 Da, Thermo Scientific). Lane 1: Purified and concentrated Bac-IB45 after passing through Sepahrose-CL6B column. **b** Gel overlay assay of band of interest against *Listeria monocytogenes* ATCC 7644

by *B. thuringiensis* and Bac14B of 20 kDa from *Bacillus subtilis* (Ge et al. 2009; Kamoun et al. 2011; Hammami et al. 2012). In the current study, the estimated molecular mass of Bac-IB45 was approximately 20.5 kDa. The homogeneity level of purified Bac-IB45 was evident by the presence of a single protein band (Fig. 3a). The band of interest was further confirmed by gel overlay assay which indicated that the inhibition of *L. monocytogenes* by purified Bac-IB45 (Fig. 3b).

Comparison of Bac-IB45 with Commercially Available Bacteriocins

The efficiency of Bac-IB45 was compared with other commercially available bacteriocins. The data revealed that nisin is the only commercially available bacteriocin which are currently used against *L. monocytogenes* in food products. Therefore, minimum inhibitory concentration (MIC) of Bac-IB45 was compared with the MIC of nisin. Results showed that the MIC of Bac-IB45 was 80 μg mL^{-1} against *L. monocytogenes* estimated by tube dilution method. In contrast, Martinez et al. (2016) reported that the commercially available nisin (Nisaplin®) was effective against *L. monocytogenes* in the MIC of 0.5 μg mL^{-1}. In another study, 100 μg mL^{-1} of nisin required to inhibit the growth of *L. monocytogenes*. (Malheiros et al. 2012). Moreover, Campion et al. (2013) reported that 12.5 μg mL^{-1} of nisin A was found to be inhibitory to *L. monocytogenes*. However, it was noticed from the comparison that the MIC of the commercially available nisin is low as compared to the MIC of Bac-IB45 but due to the limitations of nisin, Bac-IB45 could be a drug of choice in alkaline foods preservation. Moreover, Bac-IB45 has a broad antimicrobial potential as compared to nisin.

Effect of Physicochemical Parameters on Bac-IB45

It is necessary to completely characterize a newly discovered bacteriocins for their effective use and the better understanding of their mode/mechanism of action. Therefore, in this study various physical and chemical parameter were considered and their influence on the stability of Bac-IB45 was studied.

Thermal, pH and Storage Stability of Bac-IB45

The influence of pH, temperature and storage conditions was studied on the antibacterial activity of Bac-IB45. The effect of various temperatures (50–100 °C) was determined on Bac-IB45 and its antibacterial activity was calculated as the percent residual activity. Results revealed that Bac-IB45 was highly thermostable in nature and retained its antibacterial activity even at extreme high temperatures (Fig. 4a). Remarkably, it was noticed that even after exposure to 100 °C for 3 h it possesses 60% of its antibacterial activity. Moreover, it cannot only survive at the 100 °C but it remains

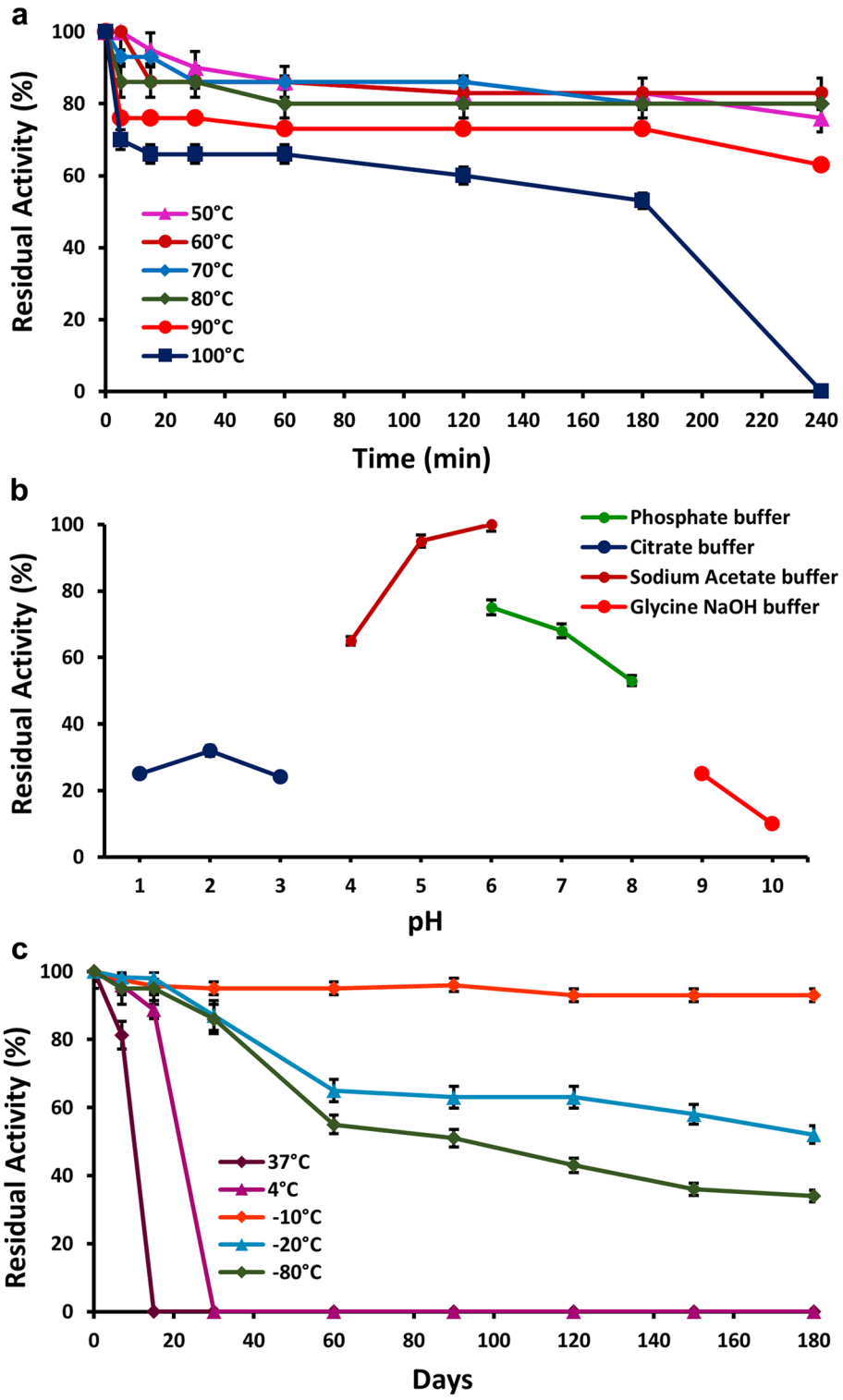

Fig. 4 Residual antibacterial activity of Bac-IB45 produced by *Lactobacillus plantarum* KIBGE-IB45 after treatment with various parameters **a** Effect of different temperatures **b** Effect of pH **c** Effect of storage conditions on the stability of Bac-IB45. The results were statistically analyzed using student's *t* test. Each value is a mean of three individual experiments and the error bars represent the standard deviation of the mean (n = 3; *p*-value < 0.05)

stable at 121 °C for 15 min under high pressure (50 psi). The thermostability of Bac-IB45 could be due to the presence of higher concentration of proline, hydrogen bonds, salt bridges, sequence and structure of the protein and polar surface deposits responsible for the stability of proteins at higher temperatures. These factors could also be accountable for the optimum antibacterial activity of proteins at higher temperatures (Kumar et al. 2000). In another study, LAB bacteriocin MN047 A (BMA) produced by *Lactobacillus crustorum* MN047 retained 90% of its antibacterial activity at 121 °C for 30 min (Yi et al. 2016a). On the other hand, Zhang et al. (2018) also reported the thermal stability of bacteriocin Lac-B23 from *Lactobacillus plantarum* J23 at 25 °C to 100 °C, but the antimicrobial activity was lost at 121 °C. Similarly, several other studies also reported the thermostable bacteriocins from other species. Bacteriocin from *Brevibacillus laterosporus* DS-3 tolerate extreme temperatures at 100 °C or 121 °C for 60 and 15 min, respectively (Odah et al. 2019). Bacteriocin BL8 was isolated from *Bacillus licheniformis*, it is also stable at 100 °C for 30 min (Smitha and Bhat 2013). Another thermostable bacteriocin, Bac-GM100 was found to be stable at 120 °C for 20 min (Ghadbane et al. 2013). However, there are also some reports, which showed that some of the bacteriocins lost their property of thermal stability upon purification (Kamoun et al. 2005). In contrast, Bac-IB45 was found to be stable after various purification steps. Thus, Bac-IB45 could be an ideal candidate for food processing industries that involves the preservation and processing of food products at high temperatures.

The thermostable Bac-IB45 was also found to be stable under broad pH range. Optimum antibacterial activity of Bac-IB45 was observed at pH 6.5 (Fig. 4b). At alkaline pH 9.0, only 25% of antibacterial activity was observed, however, Bac-IB45 retained 70% of its antibacterial activity at pH 8.0 and pH 5.5, respectively. Maximum antibacterial activity of Bac-IB45 at pH 6.5 suggests its application in various food processing industries. Moreover, the stability of Bac-IB45 at alkaline pH allows the use of this bacteriocin in the food processing of alkaline foods where other bacteriocins are restricted (Jeevaratnam et al. 2005). In another study, Paracasin SD1 produced by *Lactobacillus paracasei* SD1 was showed stability in the range from 3.0 to 8.0 pH values (Wannun et al. 2014). Moreover, another LAB bacteriocin MN047 A (BMA) produced by *Lactobacillus crustorum* MN047 was found to be highly stable at various (2.0–11.0) pH ranges (Yi et al. 2016a). Similarly, Zhang et al. (2018) also reported the antimicrobial activity of lactic acid bacteriocin Lac-B23 which was produced by *Lactobacillus plantarum* J23 found to be stable at broad pH ranges (2.0 to 12.0).

To further check the long-term storage stability of Bac-IB45, it was stored at various temperatures. Results revealed that Bac-IB45 was found to be stable as it retained 90% of its antibacterial activity even after 6 months of storage at − 10 °C (Fig. 4c). However, at − 20 °C and − 80 °C the antibacterial activity gradually decreased but still some antibacterial activity was observed. Moreover, at 37 °C and − 4 °C the antibacterial activity was completely lost after 15 and 30 days of incubation, respectively. In another study, Sharma et al. (2011) reported the stability of bacteriocin from *B. subtilis* at − 20 °C up to 2.5 months. Similarly, bacteriocins produced by *L. plantarum* F1 and *L. brevis* OG1 were found to be stable for 60 days at − 20 °C (Ogunbanwo et al. 2003). Moreover, LAB bacteriocin MN047 A (BMA) from *Lactobacillus crustorum* MN047 was showed stability up to 6 months and partially inactivated after 9 months of storage (Yi et al. 2016a). In another study, Ohenhen et al. (2015) reported the storage stability of bacteriocin produced by *Lactobacillus plantarum* at − 20 °C whereas, the antimicrobial activity was lost at 4 °C.

Influence of Metal Ions, Organic Solvents, Surfactants and Proteolytic Enzymes

Antibacterial potential of Bac-IB45 was investigated in the presence of several metal ions. Result revealed that Bac-IB45 remained stable after treatment with several metal ions (Table 2). Majority of the metal ions acted as a stabilizer in the low concentrations including Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+} and Cs^{2+}. This could be due to the presence of several stress responsive genes present in Bac-IB45 which provide stability against these stresses (Blasi et al. 2012). Moreover, Co^{2+} and Zn^{2+} inhibited the antibacterial activity of Bac-IB45 at a concentration of 5 mM. Whereas, Hg^{2+} was completely inhibited the bacterial growth even at lower concentration. It may be due to the involvement of these metal ions in the blockage of the active sites of Bac-IB45.

The potential of Bac-IB45 was also determined at different concentrations of organic solvents which were non-inhibitory for the indicator strain. Result indicated that the nature of organic solvents plays an important role in the stabilization of inhibitory potential of Bac-IB45. In the presence of methanol and DMSO, the antibacterial activity of Bac-IB45 was remained stable while, ethanol and isopropanol suppressed the inhibitory effect of Bac-IB45. However, formaldehyde was completely inhibited the antibacterial activity of Bac-IB45 (Table 2). These inhibitory and suppressing effects of organic solvents might be due to the structural changes of a protein and the modifications in non-covalent interactions. On the other hand, stability of various proteins depends on the presence of disulphide bonds (Ogino et al. 2001). Alam et al. (2011) also suggested the probability of hydrophobic nature of BLIS produced by *B. subtilis* BS15, as it showed resistant to several organic solvents except ethanol. In another study, Khalil et al. (2009) stated that the stability of a bacteriocin from *B. megaterium*-19 after

Table 2 Effect of metal ions, organic solvents, surfactants and proteolytic enzymes on the antibacterial potential of Bac-IB45 produced by *Lactobacillus plantarum* KIBGE-IB45

Metal ions[a]	% Relative antibacterial activity		
	1 mM	5 mM	10 mM
Control	100	100	100
Cs^{2+}	100	100	95
Ni^{2+}	100	100	66
Co^{2+}	100	Nil	Nil
Ca^{2+}	100	100	86
Zn^{2+}	100	Nil	Nil
Mn^{2+}	100	100	Nil
Cu^{2+}	100	100	60
Mg^{2+}	100	100	66
Fe^{2+}	100	100	30
Hg^{2+}	Nil	Nil	Nil

Organic solvents[b]	% Relative antibacterial activity	
	20 mM	50 mM
Control	100	100
Ethanol	75	Nil
Methanol	100	83
Isopropanol	75	Nil
Chloroform	91	Nil
Formaldehyde	Nil	Nil
DMSO	100	100

Surfactants[c]	% Relative antibacterial activity	
	20 mM	50 mM
Tween 20	100	100
Tween 80	83	Nil
SDS	100	92
CTAB	16	Nil
Triton-X 100 (0.05 mM)		Nil

Proteolytic enzymes[d]	% Relative antibacterial activity
Control	100
Protease	50
Lysozyme	25
Pepsin	Nil
Proteinase K	Nil

Each value is a mean of three individual experiments (n = 3)

Nil = No antibacterial activity

[a]Metal ion used as chloride salts

[b]None of the surfactants alone (−ve control) in the above-mentioned concentration inhibited the indicator strain except Triton X-100

[c]None of the organic solvents alone (−ve control) in the above-mentioned concentration inhibited the indicator strain

[d]Final Concentration of each enzyme: 1 mg mL^{-1}

treatment with ethanol and hexane might be due to the presence of lipid moieties.

Furthermore, the antibacterial potential of Bac-IB45 was determined in the presence of various anionic and cationic surfactants. In this study, non-inhibitory concentrations of the surfactants against *L. monocytogenes* were selected except for triton X-100 as it inhibited the antibacterial activity of Bac-IB45 even in the lowest concentration of 0.05 mM. Results revealed that most of the surfactants stabilized the antibacterial activity of Bac-IB45 (Table 2). This property of Bac-IB45 makes it an ideal candidate to be used as an antibacterial agent in domestic and personal care products. Similarly, Malini and Janakirama (2012) also reported the stability of a bacteriocin from *Lactobacillus acidophilus* in the presence of anionic and cationic surfactants.

Although most of the bacteriocins are usually sensitive to proteolytic enzymes. Hence, the effect of different proteolytic enzymes on Bac-IB45 showed that 50% and 25% loss in antibacterial activity when treated with protease and lysozyme, respectively. Whereas, the proteinase-K and pepsin were completely inhibited the antibacterial activity of Bac-IB45 (Table 2). A significant decrease in the antibacterial potential of Bac-IB45 was observed after treatment with proteolytic enzymes which confirms the proteinaceous nature of bacteriocin. This loss in antibacterial activity might be due to the presence of glycosidic moieties present in Bac-IB45 which is accountable for its inhibitory potential. Similarly, Oman et al. (2011) reported a research on sublancin (glycopeptides) and noticed that the glycosylation is essential for its antibacterial activity. In another study, Lactococin and enterocin were inactivated by ά chymotrypsin and proteinase K (Elotmani et al. 2002).

Antibiotic Susceptibility Testing of Multidrug Resistant Bacteria

Disk diffusion assay was used to determine the effect of various antibiotics on the multidrug resistant bacteria. Results revealed that the tested bacterial strains were found to be multidrug resistant as most of the bacteria showed resistance to commercially available broad-spectrum antibiotics (Table 3). Therefore, in the current study theses multidrug resistant bacterial strains were used to evaluate the efficiency of Bac-IB45 as a natural antibacterial agent.

Antimicrobial Spectrum of Bac-IB45

After complete purification and characterization, antimicrobial spectrum of Bac-IB45 was evaluated against various food borne and multidrug resistant bacterial and fungal strains. Result revealed that Bac-IB45 showed broad antimicrobial spectrum as it was not only inhibited various Gram positive, Gram negative food borne and multidrug resistant pathogens but also some fungal pathogenic strains (Table 4). Similarly, several studies were also reported that the LAB bacteriocins have a broad inhibitory action against various pathogenic bacterial strains (Simova et al. 2009; Goh and Phillip 2015; Lv et al. 2018). Adebayo and Aderiye (2010) also reported that LAB bacteriocins have a prominent antifungal activity against *Penicillium* and *Aspergillus* species. Whereas, Zhao et al. (2013) reported the antifungal potential of antimicrobial peptides produced by *Bacillus* against various molds includes: *Botrytis cinerea, Aspergillus niger* and *Pythium*.

Table 3 Antibiotic susceptibility patterns of multidrug resistant bacteria isolated from various sources

Multidrug resistant bacteria	Commercial antibiotics									
	IPM	DOR	FEP	VA	AMP	CIP	OX	DA	TOB	ETP
Listeria monocytogenes ATCC 7644	S	S	S	R	R	R	R	S	S	R
Pseudomonas aeruginosa ATCC 27,853	R	S	S	R	R	R	R	S	S	S
Methicillin resistant staphylococcus aureus KIBGE-IB23	S	S	S	R	R	R	R	R	R	R
Bacillus cereus ATCC 11,778	S	S	S	R	R	S	R	R	R	S
Escherichia coli ATCC 8739	R	S	S	R	R	R	R	R	S	S
Enterococcus faecalis ATCC 29,212	S	S	S	R	R	R	R	R	S	S
Salmonella typhimurium ATCC 3632	S	S	S	R	R	S	R	R	R	S

The test was performed in triplicates and the results are the mean of three individual experiments

S Sensitive, *R* Resistant, *IPM* Imipenem, *DOR* Doripenem, *FEP* Cefepime, *VA* Vancomycin, *AMP* Ampicillin, *CIP* Ciprofloxacin, *OX* Oxacillin, *DA* Clindamycin, *TOB* Tobramycin, *ETP* Ertapenem

Table 4 Antimicrobial spectrum of Bac-IB45 produced by *Lactobacillus plantarum* KIBGE-IB45 against different food borne and multi-drug resistant bacterial and fungal pathogens

Indicator Strains	Sources	Arbitrary units (AU mL^{-1})
Listeria monocytogenes	ATCC 7644	80
Micrococcus luteus	KIBGE-IB20	160
Pseudomonas aeruginosa	ATCC 27,853	80
Methicillin resistant staphylococcus aureus	KIBGE-IB23	160
Bacillus cereus	ATCC 11,778	80
Escherichia coli	ATCC 8739	80
Enterococcus faecalis	ATCC 29,212	40
Aspergillus terreus	KIBGE-IB-35	40
Aspergillus flavus	KIBGE-IB34	20
Salmonella typhimurium	ATCC 3632	−ve
Bacillus licheniformis	KIBGE-IB3	−ve
Aspergillus niger	KIBGE-IB-36	−ve
Aspergillus fumigatus	KIBGE-IB33	−ve

–ve: resistant

The experiment was performed in triplicates and each value is a mean of three individual experiments

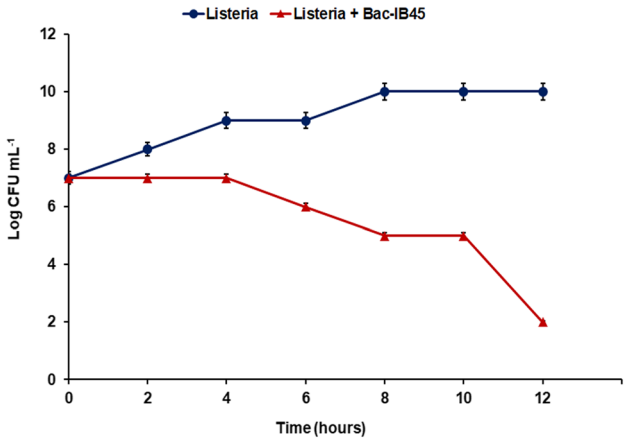

Fig. 5 Bactericidal mode of action of Bac-IB45 on the growth and survival of *Listeria monocytogenes* ATCC 7644. The experiment was performed in triplicates and the error bars represent the standard deviation of the mean (n = 3; *p*-value < 0.05)

Mode of Action of Bac-IB45

For the applications of bacteriocins in pharmaceutical and food industries, there is a need to elucidate its mode and mechanism of action. Generally, the antimicrobial compounds used in antimicrobial therapies are either bacteriostatic and/or bactericidal in nature. In this study, time-kill assay revealed that the Bac-IB45 has a bactericidal mode of action against *L. monocytogenes* ATCC 7644 in a lethal concentration of 80 µg mL^{-1}. Moreover, the growth of *L monocytogenes* was completely inhibited after 12 h of treatment (Fig. 5). In order to confirm the bactericidal mode of Bac-IB45, the re-growth of the *L. monocytogenes* was also checked. Result showed that no growth of *L. monocytogenes* was observed after 24 h of incubation, which confirms the bactericidal mode of action. This bactericidal effect might be due to the release of ions and ATPs from bacterial cell because of the increase permeability of the cytoplasmic membrane that ultimately caused death of the cells. (Gálvez et al. 1990). Moreover, a substantial decline in the viability of cells and the optical density was examined after 6 h of treatment which indicated the lysis of the bacterial cells due to the activation of autolysin. In numerous other studies bactericidal mode of action of sakacin C2, plantaricin ZJ008 and bacteriocin GM3 was reported (Gao et al. 2010; Zhu et al. 2014; Devi et al. 2016).

Effect of Bac-IB45 on *L. monocytogenes* under Scanning Electron Microscope

In order to confirm the mechanism of Bac-IB45 on the structural integrity of *L. monocytogenes* scanning electron microscope was used. After the treatment with Bac-IB45, structural changes were observed on the cell membrane of *L. monocytogenes*. Result revealed that untreated cells showed typical features of rod-shaped with the smooth surface while, the treated cells were deformed, shrunk and ruptured after 8 h. It might be due to the formation of pores and the release of the hydrophilic molecules from the treated cells (Fig. 6). Bac-IB45 was found to be highly effective on the structure and membrane integrity of *L. monocytogenes* which may leads to death of the cell that was previously observed in time-kill assay. Generally, morphological changes were observed on the cell surface of the sensitive cells after the treatment with bacteriocins (Bajpai et al. 2013). In this study, effect of Bac-IB45 on cell membrane of bacteria was similar as reported by other bacteriocins for instance: plantaricin K25 and lactocin LXA (Wen et al. 2016; Yi et al. 2016b). Similarly, in another research scanning electron microscopy showed the smooth cell membrane of untreated *E. coli* whereas, treated cells with bifidocin A displayed wrinkled surface (Liu et al. 2016). It is also reported that the plantaricin JY22 damaged not only the cells but also the spores of *B. cereus* (Lv et al. 2018).

Assessment of Cytotoxic Effect of Bac-IB45 on Fibroblast Cell Line

The cytotoxic effect of Bac-IB45 was examined against NIH/3T3 fibroblast cell line using MTS assay. The cells were incubated with various concentrations of Bac-IB45 including 2 × MIC, MIC and 0.5 × MIC (Sharma et al.

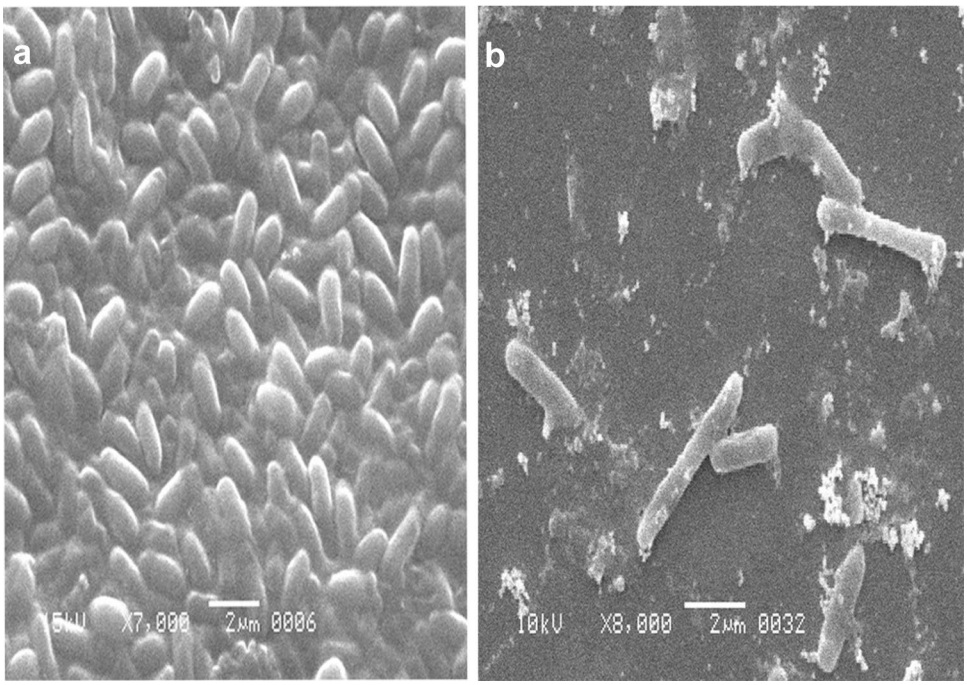

Fig. 6 Scanning electron micrographs showing morphological changes in *Listeria monocytogenes* ATCC 7644 after treatment with 80 μg mL^{-1} concentration of Bac-IB45 **a** Untreated *Listeria monocytogenes* ATCC 7644 **b** *Listeria monocytogenes* ATCC 7644 after 8 h of treatment with Bac-IB45

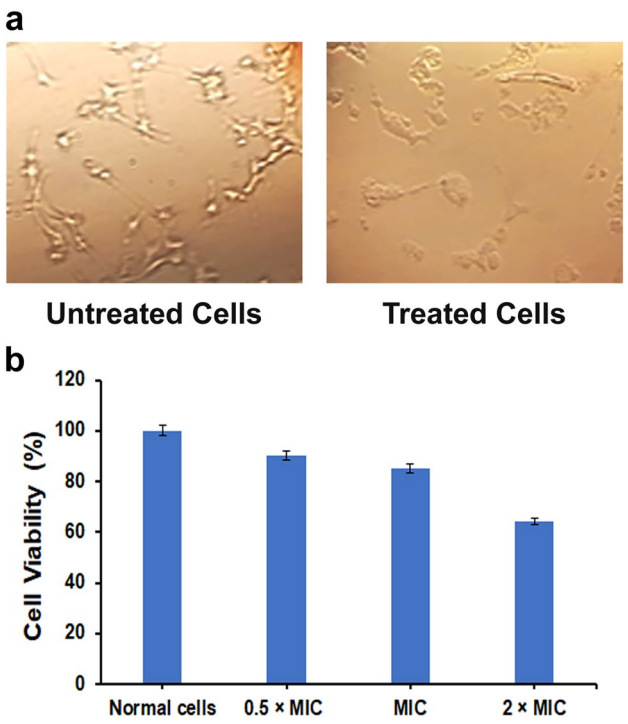

Fig. 7 **a** The morphological analysis of untreated and treated NIH/3T3 cells with Bac-IB45 (80 μg mL^{-1}) using optical microscope **b** Effect of different concentrations of Bac-IB45 on the viability of NIH/3T3 cells. Cells were treated with 2 × MIC (160 μg mL^{-1}), MIC (80 μg mL^{-1}), and 0.5 × MIC (40 μg mL^{-1}) of Bac-IB45 for 24 h at 37 °C. The results were statistically analyzed using student's *t* test. Each value is a mean of three individual experiments and the error bars represent the standard deviation of the mean (n = 3; *p*-value < 0.05)

2017). It was noticed that the cell viability was concentration dependent. The morphological changes were examined in the treated cells compared to untreated cells (control) using an optical inverted microscope. After treatment with the minimum inhibitory concentration of Bac-IB45 (80 μg mL^{-1}), most of the cells remain viable. There is no significant decrease in the cell viability was observed (Fig. 7a). Result revealed that 85% of the cells found to be viable after treatment with the minimum inhibitory concentration (80 μg mL^{-1}) of Bac-IB45 Whereas, 90% and 64% cell viability was noticed in 0.5 × MIC and 2 × MIC, respectively (Fig. 7b). These results suggested the non-cytotoxic nature of *Bac-IB45*. Therefore, it can be used as a natural bio preservative in the food industry and as alternative therapeutics in pharmaceutical industry. Similarly, Amrita and Kadirvelu (2019) reported that the bacteriocin from *Lactobacillus plantarum* SJ33 exhibited non cytotoxic effect on IEC-6 and HEK-293 cell lines. In another study, bacteriocin produced by *L. sakei* GM3 showed maximum survival of human adenocarcinoma cancer (HT29) cell line (Avaiyarasi et al. 2016).

Conclusions

In this study, Bac-IB45 from *L. plantarum* KIBGE-IB45 was purified and characterized. Bac-IB45 has a broad inhibitory spectrum against various multidrug resistant and food borne pathogens. SEM results revealed that Bac-IB45 showed drastic effect on the structural integrity of

L. monocytogenes which may leads to cell damage. The cytotoxic analysis on fibroblast cell line indicated that Bac-IB45 was nontoxic to the cells in its minimal inhibitory concentration. Therefore, Bac-IB45 could be used as a bio-preservative in food products and as a next generation antibiotic in pharmaceutical industries. In future, more studies will be conducted on the structure, cytotoxic analysis on other cell lines and the molecular mechanism of Bac-IB45.

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Compliance with Ethical Standards

Conflicts of interest All the authors declared that they have no conflict of interest.

Ethical Approval This manuscript does not contain any research involving human participants and/or animals.

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