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PRODUCTION AND PRELIMINARY CHARACTERIZATION OF BACTERIOCIN FROM *ENTEROCOCCUS FAECIUM* AGAINST *LISTERIA MONOCYTOGENES*

PAVANA JYOTHI CHERUKURI*, REETHU NARAYANAN AND RAJANI CHOWDARY AKKINA

Department of Microbiology and Food Science & Technology GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, India

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Abstract – The emergence of antibiotic resistance pathogens and limitation by chemical preservatives has raised to search for novel, safe, natural food preservatives. Bacteriocins are small molecular weight peptides derived from lactic acid bacteria are used as natural and safe food preservatives. The aim of the present study is to isolate a potential bacteriocin producing probiotic lactic acid bacteria, among 157 strains of lactic acid bacteria isolated from various foods, *Enterococcus faecium* KY711240 BL7 isolated from non fermented south Indian vada (a snack item) batter was selected based on potential bacteriocin production against *Listeria monocytogenes*. This bacteriocin is stable at autoclaving temperature (121 ^{o}C/ 15 minutes) active over pH range pH 3 – 9, sensitive to proteolytic enzymes and resistant to amylase and lipase enzymes.

INTRODUCTION

Listeria monocytogenes is a challenging food borne pathogen in food industries. It is ubiquitous in nature and can thrive in cold, humid, low oxygen environment, hence a persistent contaminant in convenience and refrigerated foods (Dongyou, 2008). It causes listeriosis, a food-borne disease characterized by potentially fatal septicemia in immune-compromised individuals, meningitis in newborns and abortion in pregnant women (Juan *et al*., 2017). These characteristics of *Listeria monocytogenes* have drawn attention of food microbiologists to develop novel food preservative to overcome the current challenge. According to European Food Safety Authority, 2014 reported *Listeria monocytogenes* caused 198 deaths out of 1642 confirmed cases in 2012. United States reported 30 people killed out of 148 sickened by consuming *Listeria* contaminated cantaloupe in 2011 (Caroline *et al*., 2014).

The use of chemical preservatives controls microbial spoilage in foods but has shown deleterious health effects (Mills *et al*., 2011). Nitrates used as a preservative in foods have been associated with incidence of cancer in humans (Song *et al*., 2015). Excess of antibiotic usage in foods havebeen restricted and prohibited globally. Demand for minimally processed foods has opened a new dimension to investigate potential, natural and safe bio-preservatives. Metabolic products from probiotic lactic acid bacteria such as organic acids and bacteriocins can be potential biopreservatives for convenience foods such as Ready to eat/ Ready to serve/Heat and eat food products (Reis *et al*., 2012).

Bacteriocins are low molecular weight bioactive peptides displaying antimicrobial activity and can be used as bio-preservatives. They are active against pathogenic bacteria, exhibiting either narrow or broad spectrum of activity. Bacteriocins have a numerous positive attributes, such as biodegradable, thermo-stable and active over a wide range of pH. Bacteriocins generally act through inhibition of cell wall synthesis or depolarization of the target cell membrane (Ana *et al*., 2017). Bacteriocin can be incorporated into foods through three different routes a) direct application of purified bacteriocin in packaging material b) bacteriocin-based ingredients from fermented foods) incorporation of bacteriocin producing LAB (Lactic Acid Bacteria) in foods (Schillinger *et al*., 1996).

According to the WHO definition Probiotics "a

live microorganism which when administered in adequate amount confers health benefits to host". Probiotics provide either nutritional or therapeutic health benefits to host (Vasiljevic *et al*., 2008). Majority of Lactic acid bacterial genera used as starter culture in food industry due to their GRAS status (generally recognized as safe) and play a significant role in maintaining the microbial balance of intestine by inhibiting harmful pathogens. Manufacturers of functional foods particularly probiotic foods, are under constant pressure to screen efficient probiotic strain. The selection criteria required for LAB to function as probiotic (i) exert a benecial effect on the host, (ii) survive the harsh environment of gastrointestinal tract, (iii) high cell count in food products and remain viable throughout the shelf-life of food product and (iv) produce antimicrobial substances (organic acids, bacteriocins) against pathogens (Parvez *et al*., 2006). The current study was focused on isolation, identification and characterization of antilisterial bacteriocin producing probiotic strain.

MATERIALS AND METHODS

Microorganisms and culture conditions

Four standard lactic acid bacteria were obtained from IMTECH Chandigarh, India (MTCC 1423 *Lactobacillus casei*, MTCC 9495 and 9496 *L.plantarum*, MTCC 3041 *Lactococcus lactis*) and four strains from Agriculture Research Service, USA (B-763 *L.delbruki*, B-1949 *L.s alivarius*, B-14853 *L.ruminis and* B-4560 *L.paracasei)*. They were revived aerobically in MRS broth, incubated at 37 °C for 48 hours. Indicator strains MTCC 657 *Listeria monocytogenes, isolated Listeria monocytogens* from spoiled meat were cultivated aerobically in brain heart infusion broth at 37 °C for 24 hours.

Along with standard strains, lactic acid bacteria were isolated from different samples such as milk, dairy products and traditional fermented (idli, appam) and non-fermented (vada) batters, fresh, fruits and vegetables. These samples were serially diluted 6 fold. 100 µL was spread on MRS agar and incubated for 48h at 37 °C. A total of one fifty seven well isolated cells with circular, raised and entire margin were selected and purified using pure culture techniques. Along with standard strains, pure isolates were screened for Gram characteristic and catalase activity. Only Gram positive and catalase negative strains were selected and screened for antimicrobial activity against two *Listeria monocytogens* strains.

Initial screening of antimicrobial activity

For bacteriocin production by isolated and standard strains using 100 mL MRS broth pH (6.5) was inoculated at a level of 10^4 CFU/mL and incubated at 37 °C. The antimicrobial activity of standard and isolated lactic acid bacteria was determined by agar well diffusion method. Lactic acid bacteria grown in MRS broth for 16 to 18h was centrifuged at 8000 rpm, 10 min at 4 °C. Cell free supernatant (CFS) was collected and filtered through 0.45µm membrane filter. Overnight culture of indicator strains (*Listeria monocytogenes*) was seeded on brain heart infusion soft agar (0.7% agar). Wells of 5 mm diameter were punched on soft agar, filled with 50 µL of CFS and incubated at 37 °C for 24 hr and measured inhibition zones.

Qualitative Determination of bacteriocin

CFS of Lab was adjusted to pH 6.5 with 1N Na OH and treated with catalase enzyme and incubated for 1h for screening of antimicrobial activity, then treated with protease enzyme (1 mg/mL) with 1h incubation to confirm as bacteriocin.

Ammonium sulphate precipitation

Cell free supernatant was saturated to 30% by slow addition of ammonium sulphate with constant gentle stirring overnight at 4°C. Saturated crude supernatant was centrifuged at 12,000 rpm for 25 min at 4°C. Precipitate obtained was collected and dissolved in 50mM phosphate buffer (pH 6.5) and 50 µL was assayed for antibacterial activity (AU/ml) against *Listeria monocytogenes*. The remaining solution was saturated to next levels (35%, 40%, 45%, 50%, 55%, 60%, 65% and 70%) and bacteriocin activity was measured by each saturation levels.

Dialysis

Dialysis membrane (SIGMA) of molecular weight cut off (MWCO) 2000Da of suitable length was boiled in 2% (w/v) sodium bicarbonate ($NaHCO_3$) and 0.05% EDTA (Ethylene diaminetetraaceticacid) solution, cooled and again boiled for 10 minutes in sterile distilled water. One end (bottom end) of dialysis membrane was sealed and through other opening precipitate dissolved in 50mM sodium phosphate buffer (pH7) was added and again securely sealed. Membrane was placed in beaker containing 50mM sodium phosphate buffer and placed on magnetic stirrer to allow gentle stirring of

dialysis membrane. Dialysis was carried at 4 °C with frequent changes of buffer to remove ammonium sulphate from bacteriocin which may hinder further purification experiments. After completion of dialysis an aliquot was assayed for protein concentration at A_{280} (blank –sodium phosphate buffer). Bacteriocin activity (AU/mL), specific activity, purification fold and yield were calculated.

Cation exchange chromatography

The bacteriocin solution obtained after dialysis was further purified by cation exchange chromatography using CM sephadex 25 (SIGMA).

Column packing

Glass column (1x20cm) was thoroughly cleaned with distilled water. CM sephadex 25 cation exchange resins (Sigma-Aldrich, USA) was suspended in 0.05M (50mM) sodium phosphate buffer pH 7 (binding buffer) and swollen by boiling in water bath for 2h at 90 °C. Vague solution was discarded. Same buffer was again added, gently mixed, obtained vague solution was again discarded. This process was repeated until solution was clear.

Hydrated gel suspension was carefully loaded to the glass column (1X30cm) without air bubbles and allowed to settle while maintaining a slow flow rate of phosphate buffer through the column. Column was equilibrated by passing the 0.05M sodium phosphate buffer pH 7 through the column bed until the pH of eluent is same as the incoming buffer.

Sample application and elution

Column was loaded with 5 mL of dialysed partially purified samples. After the complete entry of sample into the column, the peptides were eluted using linear gradient elution (20 mL) 0.1M, 0.5M and 1 M NaCl solution prepared in 0.05M sodium phosphate buffer pH 7 with a flow rate of 1ml/ minute. All the collected fractions were filtered sterilised and examined for bacteriocin activity against *Listeria monocytogenes*. Active fractions were pooled, assayed for specific activity, protein concentration and yield.

Characterization of partially purified bacteriocin

Partially purified sample was further characterised for its stability (enzymes, temperature, pH) molecular weight determination by SDS-PAGE, mode of action.

Effect of enzymes, temperature, pH on bacteriocin activity

CFS was treated with various enzymes (proteinase k, trypsin, pepsin, lipase and α-amylase) at a concentration 1 mg/mL and incubated for 1h and bacteriocin activity was determined (AU/ml) by critical dilution method. To determine thermal stability of bacteriocin, the CFS was heated in water bath at various temperatures (70 °C, 80 °C, 90 °C, 100 °C for 30 min and autoclaved at 121 °C for 15 min separately) and to determine the pH sensitivity the pH of CFS was adjusted from 2 to 10 with 1N HCl and 1N NaOH incubated for 1h, finally readjusted the pH 6.5 and bacteriocin activity was assayed.

Mode of action of bacteriocin

To study the mode of action of bacteriocin, CFS (1ml) adjusted to pH 6.5 was added to 9 mL brain heart infusion broth inoculated with indicator strain *Listeria monocytogenes* (500 µL) for 24h at 37 °C. At 0h, 4h, 8h, 24h, viable counts of *Listeria monocytogenes* was measured in brain heart infusion agar by Colony Forming Units (CFU).

RESULTS AND DISCUSSION

Isolation and screening of bacteriocin producing probiotic organism

A total of one fifty seven strains were isolated from dairy, fermented and non fermented food products, fresh vegetables and fruits. From this, seventy nine Gram positive and catalase negative lactic acid bacteria were selected for bacteriocin production. These isolates along with eight standard MTCC and ARS cultures were screened for their antimicrobial activity against *Listeria monocytogenes*. Among these strains eleven strains have shown potential antimicrobial activity against *Listeria monocytogenes* (Table 1).

The pH of CFS of these eleven strains was adjusted to 6.5 and treated with catalase enzyme separately. Among these strains only four isolated strains BL7, BL3, S1 and V10 exhibited antagonism against *Listeria monocytogenes*. No inhibition zones were found after treating with protease enzyme. Thus the inhibition by BL7, BL3, S1 and V10 strains against *Listeria monocytogenes* was confirmed to be mediated through bacteriocin. Among four bacteriocin producing lactic acid bacteria strains *E. faecium* KY711240 have showed maximum bacteriocin (1200AU/mL) production. Many

Table 2. Biochemical characteristics of *Enterococcus faecium KY11240*

Characteristic	Indole	Methyl red	Voges proskauer	Citrate	Casein	Esculine hydrolysis	H_2S gas production	Glucose	Sucrose	Lactose	Mannose	Galactose	Maltose	Treha-lose	Ribose	Xylose	Raffinose	Rhamnose	Melizitose	Arabinose
E.faecium KY711240 BL7	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+

*"+" – positive; "-" negative"

researchers have reported *Enterococcus faecium* was able to produce bacteriocin against *Listeria sps* (Gaaloul *et al*., 2014; Ndlovu *et al.,* 2015).

Effect of enzymes, pH and thermal stability on bacteriocin activity

CFS of *Enterococcus faecium* KY711240 BL7 was subjected to different stability tests. Bacteriocins are small molecular weight peptides easily get degraded in the gastrointestinal tract by proteases as a result they cannot alter the microflora of the intestine. Heat stability of bacteriocin can be used with thermal processing of food products for preservation. Bacteriocin activity must be stable in acidification of foods, used to reduce spoilage and enhance flavor to the food product.

Bacteriocins have shown stable activity with lipase and amylase and sensitive to proteinase k, trypsin and pepsin. This clearly indicates that all bacteriocins are proteinaceous in nature and do not contain carbohydrate or lipid molecule. Similar findings were reported by Badrinath *et al*. 2011 on enterocin P. Bacteriocin from *Enterococcus faecium* KY711240 was stable at autoclaved conditions of 121 ^{0}C for 15 min and stable over broad pH range (2 to 9) (Table 4). Majority of bacteriocins from *Enterococcus faecium* is stable upto 100 ^{0}C, but lost activity at 121 ^{0}C, and stable at wide pH range 2-9 (Shin *et al*., 2008, Gaaloul *et al*., 2014).

Modes of action of bacteriocins have shown either bactericidal or bacteriostatic activity, depends on many factors such as type of indicator strains, physiological state, dose and purity of bacteriocins (Cintas *et al*., 2001). Mode of action of bacteriocin from isolated *Enterococcus faecium* KY711240 shows bactericidal activity. A decrease in viable number of *Listeria monocytogenes* was observed from 4h and no viable counts were observed after 24h of incubation. Enterocin from *Enterococcus faecium* exhibit bacteriocidal activity against *Lactobacillus pentosus*, *Listeria ivanovi* and *Pseudomonas aeruginosa*. (Ndlovu *et al*., 2015; Gaaloul *et al*., 2014; Todorvov *et al*., 2013).

Antibacterial activity of bacteriocin on storage at two different temperature

Partially purified bacteriocin from *Enterococcus faecium* was stored in incubator at 37 ^{o}C and in refrigerator 4 ^{o}C for one month. Samples (50µL) were taken out once in a week and tested for antibacterial activity against indicator bacteria *Listeria monocytogenes* by agar well diffusion method.

Ammonium sulphate precipitation

Cell free supernatant from *E. faecium* KY11240 was subjected to ammonium sulphate saturation from 30% to 70%.

No appreciable inhibition was observed on saturation upto 40%. Antimicrobial activity increased with increasing saturation from 45% to 70% with maximum antimicrobial activity (AU/mL) obtained at 70% saturation with specific activity 560137 AU/mg in comparision with crude supernatant (specific activity 68266 AU/mg). Bacteriocin precipitated with 70% saturation resulted 8.2 fold purification of bacteriocin. Saturation above 70%, i.e. 80% showed decreased inhibition and there after on further saturation no inhibition was observed (Fig. 1).

Ammonium sulphate precipitation has been successfully used by many researchers to precipitate bacteriocin. Ge *et al.,* (2016) precipitated bacteriocin produced by *Lactobacillus paracasei* with 70% ammonium sulphate. Specific activity increased to 410.34 from 336.10 with 94.62% of recovery.

Similarly Ouda *et al.,* (2014) obtained maximum bacteriocin activity of 22880AU/mL on 60% ammonium sulphate saturation. Enterocin NM2 was purified to 160 fold and specific activity 80000 on saturation with 40% ammonium sulpahte saturation (Enan *et al.,* 2014).

Bacteriocin produced from *Bacillus subtilis* BTFK101 and *Bacillus licheniformis* BTHT8 showed

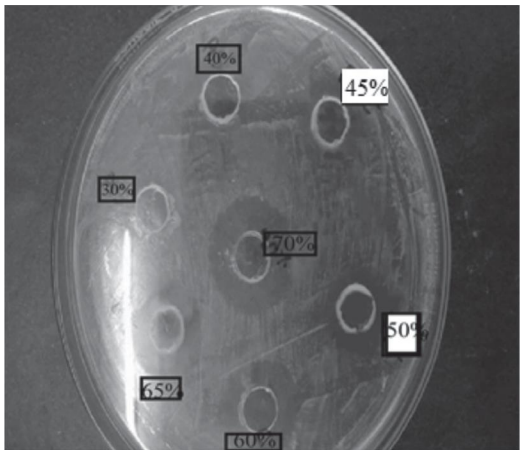

Fig. 1. Zones obtained at various Ammonium Sulphate saturation

antimicrobial activity on saturation with 30 – 60% ammonium sulphate, with purification fold of 4.723 and 6.128 respectively. Precipitation of bacteriocin from *L. pentosus* K2N7 by 60 % ammonium resulted in 58-fold concentration and 99.2% recovery with specific activity 78.31 AU/mg (Nisit Watthanasakphuban *et al.,* 2016).

The precipitate obtained was dissolved in 10 mL 50mM phosphate buffer (pH 7) and dialysed against same buffer for 24h. Dialysed fraction was applied on cation exchange column. Dialysis was done to remove unwanted compounds (ammonium sulphate) from precipitate using semipermeable membrane that allow smaller unwanted compounds to pass out and retain the peptide.

Cation exchange chromatography

Partially purified bacteriocin was passed through CM Sephadex 25 cation exchanger and bounded peptide was eluted through linear gradient elution (0.1M, 0.5M and 1M NaCl in 0.05M phosphate buffer pH 7). Bacteriocin was eluted with 1M NaCl. Fractions C68 to C73 exhibited antimicrobial activity. Specific activity was decreased from previous precipitation step (41796AU/mg) with decrease in fold of purification (0.61) and decreased yield (2.5%).

Cation exchanger purifies peptide based on charge of peptides. Peptides with net positive charge behave as cations and binds to cation exchanger (negative charge). Several researchers used ion exchanger (cation or anion) for further purification after precipitation and dialysis.

Characterization of bacteriocin

Partially purified bacteriocin from isolated *Enterococcus faecium* was characterised based on its molecular weight, heat stability, pH stability, effect of enzymes, effect of solvents on bacteriocin.

Molecular weight determination and purity

Partially purified bacteriocin obtained after cation exchange chromotography was run on SDS-PAGE to determine its molecular weight and to determine bacteriocin purity. Figure 2 shows a single peptide band on SDS PAGE gel with molecular weight of below 14 kDa.

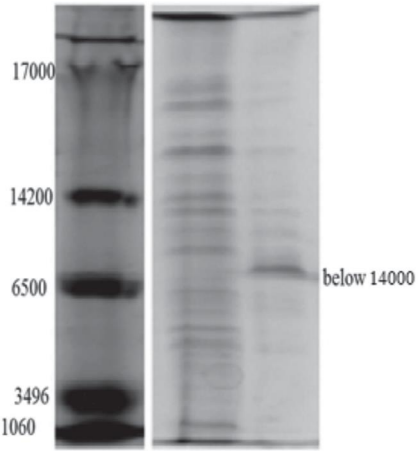

Fig. 2. SDS PAGE of bacteriocin obtained from *Enterococcus faecium*

Similar way molecular mass of various bacteriocin purified by various techniques has also been estimated by SDS-PAGE. Paracin 10kDa from *L. paracasei* (Ge *et al.,* 2016), bacteriocin from *Lactobacillus viridescence* with molecular weight 8.3kDa (Sure *et al.,* 2016) were determined by SDS-PAGE. Few researchers determined molecular weight by MALDI apart from SDS PAGE (Goh and Philip 2015; Ge *et al*., 2016; Nisit Watthanasakphuban *et al*., 2015).

Stability tests for bacteriocin

Temperature stability of bacteriocin

Purified bacteriocin was subjected to different temperature 100 °C and 121°C. Table 2 shows stability of purified bacteriocin at different temperature. At 100 °C partially purified bacteriocin activity measured 81290AU but at 121°C antimicrobial activity decreased by two fold (20480AU/mL).

The retention of antimicrobial activity of bacteriocin shows that it is heat stable generally low molecular weight bacteriocins are considered heat

Table2. Effect of temperature on activity of partially purified bacteriocin from isolated *Enterococcus faecium*

Temperature	100 ^{O}C	121 ^{O}C
Activity AU/mL	81290	20480

stable because of their simple structure. Heat stability of bacteriocin indicates it can be used with thermal processing to preserve food products (hurdle technology). Bacteriocin from *Enterococcus faecium* MTCC 5153 (Badrinath and Halami, 2011) is stable up to 100 ^{0}C, but lost activity at 121 ^{0}C. Gaaloul *et al.,* (2014) reported activity of bacteriocin even at 121 ^{0}C. Few bacteriocins such as helveticin are heat labile. This is due to their large size and complex structure.

pH stability of bacteriocins

Purified bacteriocin was adjusted to different pH values (2 – 10) for 2h. At pH 2 and 10 residual activity of bacteriocin was completely absent. At pH 3, 4, 8 and 9 activity was decreased but at pH range 5 to 7 antimicrobial activity was stable. Paracin isolated from *L. paracasei* was stable from pH 3 to 8 with maximum activity seen at pH 3 and 4. Similar results were reported by Shin *et al.,* (2008) who found bacteriocin produced from *Enterococcus* and *Pediococcus* strains to be stable at pH range 2 – 9. Increasing pH values lowered the stability of bacteriocin in finding of Ge *et al.,* (2016).

Effect of organic solvents on bacteriocin activity

Partially purified bacteria completely retained its activity in organic solvents. Results shown in Table 2. (Figure 3)

Effect of enzymes on bacteriocin activity

According to definition bacteriocin are

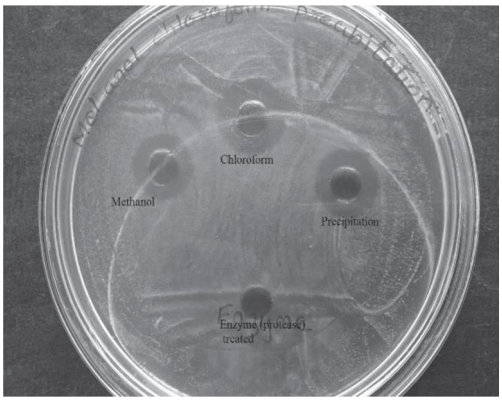

Fig. 3. Partially purified bacteriocin treated with organic solvents

proteinaceous in nature. Since bacteriocins are proteinaceous in nature they are safe biopreservatives. They easily get degraded in the gastrointestinal tract by proteases as a result they cannot alter the microflora of the intestine (Clevaland, 2001). Purified bacteriocins lost activity after incubation with proteinase k, trypsin, pancreatin and pepsin but were stable with lipase and amylase. This clearly indicates that all bacteriocins are proteinaceous in nature and do not contain carbohydrate or lipid molecule (Fig. 4).

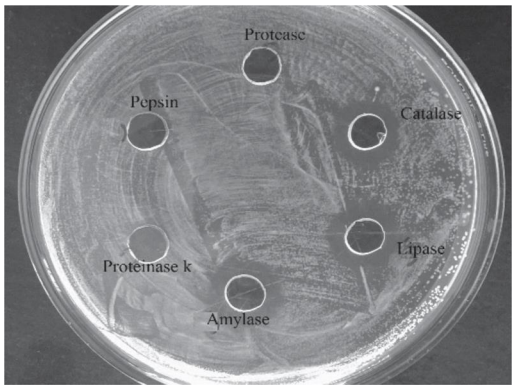

Fig. 4. Action of enymes on partially purified bacteriocin

Mode of action

Bacteriocins show either bactericidal or bacteriostatic activity on pathogenes. The mode of action of bacteriocin depends on many factors such as type of indicator strains, physiological state, dose and purity of bacteriocins (Cintas *et al.,* 2001).

A decrease in viable number of *Listeria monocytogenes* was observed from 1h and no viable counts were observed after 4h. Bactericidal mode of action has been reported by many researchers. Ndlovu *et al.,* (2015) observed complete inhibition of *Lactobacillus pentosus* by partially pure enterocin produced from two strains of *Enterococcus faecium* #6 and 112.4. Similar mode of action has been reported by Gaaloul *et al.,* (2014) for bacteriocin produced from *Enterococcus faecium* strain GGN7 against *Listeria ivanovi* and *Pseudomonas aeruginosa*. Todorvov *et al.,* (2013) also reported bactericidal activity on *Enterococcus faecium* ATCC 19443 by strains of *Lactobacillus sakei*.

CONCLUSION

In the present study bacteriocin produced from *Enterococcus faecium* KY11240 was partially purified through 70% ammonium sulphate saturation

followed by cation exchange chromatography. Bacteriocin activity obtained was 81920AU/mL. Partially purified bacteriocin was stable at temperature 121 °C, exhibited activity from pH range 3 to 9, stable in organic solvents, molecular weight determined below 14000Da.

In recent years *Listeria monocytogenes* has caused economic loss to food industry and severe food borne illness to public. Chemical preservatives trigger deleterious health issues and develop antibiotic resistance strains. Therefore to use natural preservatives is an alternative for enhancing the shelf life of food products.

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Conflict of Interest

The authors declare no conflicts of interest

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