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Purification and Characterization of Bacteriocin Produced by *Bacillus subtilis* R75 Isolated from Fermented Chunks of Mung Bean (*Phaseolus radiatus*)

Nivedita Sharma, Riti Kapoor, Neha Gautam and Ranjana Kumari*

Department of Basic Sciences, Dr. Y.S. Parmar University of Horticulture and Forestry Nauni, Solan (H.P.) 173 230, India

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Summary

Food-grade bacteria capable of producing bacteriocin with desirable preservation attributes have been isolated from traditional Indian fermented food dal vari, which has not been investigated so far. Among different isolates, *Bacillus subtilis* R75, isolated on MRS agar, exhibited antagonism against a wide range of foodborne pathogens that cause serious spoilage. Extracellularly produced bacteriocin was purified by single step gel exclusion column chromatography. The purity rate and molecular mass of 12 kDa of this compound were determined using SDS-PAGE. Activity units (AU) of bacteriocin were increased in each step of purification, reaching up to $5 \cdot 10^6$ AU/mL. The increase in the activity units directly affected the antimicrobial activity of purified bacteriocin, resulting in an increase up to 200, 333 and 175 % of the inhibition zones against indicator bacteria. Continuous decrease in the number of viable cells of microorganisms within 10 h after adding purified bacteriocin proved its bactericidal action. It withstood very high temperature, up to 121 °C, for 10 min, wider pH range, from 4.0 to 11.0, complete inactivation in the presence of proteolytic enzymes and storage stability up to 2.5 months.

Key words: bacteriocin, purification, biopreservative, antimicrobial activity, indicator bacteria

Introduction

The growing consumer demand for finding natural but effective preservation of food free of potential health risks has stimulated research in the field of biopreservation to find an attractive and alternative approach to chemical preservatives. Although many bacteria have served as food preservatives for millennia, their activity was not well known until about a decade ago. Recently scientists have developed a concept of biopreservation, *i.e.* targeting food pathogens/spoilage-causing microorganisms with the help of these prokaryotes/their metabolites and thus providing longevity and safety of food. Among biopreservatives, bacteriocin, a low molecular mass proteinaceous compound secreted by bacteria, is catching rapid attention due to its GRAS (generally recognized as safe) status without causing any adverse effect on food (*1*). Bacteriocin is believed to be safe for human consumption since it becomes inactive when treated with digestive enzymes in the stomach (*2*). In nature, each bacterium is capable of secreting new bacteriocin (*3*); however, this potential of microorganisms has largely been unexplored. So far, only one bacteriocin, nisin, has been awarded the status of food preservative by Food and Drug Administration (FDA), USA, and is being used commercially worldwide by food industries. That is why scientists need to isolate microorganisms which have not been investigated so far. In the present study, a traditional fermented food of a Himalayan state of India, dal vari, has been chosen for isolation of new strains of food-grade bacteria capable of producing bacteriocin with desirable attributes for preservation of food.

*Corresponding author; Phone: ++91 1792 252 560; Fax: ++91 1792 252 242; E-mail: niveditashaarma@yahoo.co.in, neha_mbg@yahoo.com

Materials and Methods

Isolation, screening and identification of bacteriocin-producing strains

Dal vari, fermented chunks of *Phaseolus radiatus* (mung bean), are traditional food in the part of India known as Himachal Pradesh. It is made from fermented mashed pulse of *Phaseolus radiatus* by soaking it in water, grinding and finally drying the small chunks under the sun.

Isolation

For isolation of bacteriocin, fermented material was soaked in 10 mL of distilled water at 27 °C for 24 h. After crushing in sterilized mortar with pestle, it was serially diluted in the range from 10^{-2} to 10^{-8}. Isolation of bacteria was carried out by pour plate method on nutrient agar at 37 °C for 72 h. In total, 11 different strains were isolated. These strains were screened for bacteriocin production against indicator bacteria through well diffusion method.

Indicator bacteria

The serious food-borne pathogens and spoilage-causing microorganisms like *Listeria monocytogenes*, *Staphylococcus aureus*, *Leuconostoc mesenteroides*, *Escherichia coli*, *Enterococcus faecalis*, *Lactobacillus plantarum*, *Bacillus cereus*, *Bacillus subtilis* and *Clostridium perfringens* were procured from Microbial Type Culture Collection, Chandigarh, India, to study the antagonistic pattern of the isolates. Further studies of bacteriocin activity were performed only with the most severe indicator bacteria, *i.e. L. monocytogenes*, *L. mesenteroides* and *L. plantarum*.

Screening

Screening of isolates was done through well diffusion assay as given below and the size of clear zones was recorded against the maximum number of indicator bacteria to select the best strain.

Well diffusion assay

Isolated bacterial strains were grown in nutrient broth separately, incubated at 37 °C for 3 days at 150 rpm. The cultures were centrifuged at 10 000×*g* for 5 min and the supernatant was collected for further studies. The pH of the collected supernatant was adjusted to pH=7.0 by 1 M NaOH. A volume of 1 mL of inoculum of each indicator bacteria (A=1.0 at 540 nm) was swabbed on prepoured sterilized nutrient agar plates using sterilized cotton bud. The wells of 7 mm in diameter and 5 mm deep were cut and 300 μL of culture supernatant were poured in each well. The plates were incubated at 37 °C for 24 h and the clear zones formed around the wells were measured (*4*). Out of all isolates, R75 was selected for further studies because it showed the largest inhibition zone against the maximum number of indicator bacteria.

Identification

A hyperproducer of bacteriocin, isolate R75, was identified with the help of 16S rRNA gene technique as *Bacillus subtilis.* PCR amplification was done to confirm the identity of the bacterial strains.

DNA extraction, polymerase chain reaction (PCR) and DNA sequencing

Genomic DNA was extracted by using DNA purification kit (Genei, Bangalore, India). The PCR analysis was carried out with a volume of 20 μL of mixture in a DNA gradient thermocycler (Model no. P 818070424, Astec, Fukuoka, Japan). The procedure comprised 35 cycles at 92 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min. The primer used for amplification was 16S 1375U (5′ GCAAGTCGAGCGGACAGATGGGAGC 3′) and 16S 1375D (5′ AACTCTCGTGGTGTGACGGGCGGTG 3′).

The amplified PCR product was harvested from 1 % agarose gel and purified with gel extraction and PCR purification kit (Sigma-Aldrich, St. Louis, MO, USA). The purified product was sequenced with an automated sequencer (ABI PRISM 310, Applied Biosystems, Foster City, CA, USA).

BLASTN analysis

Translated nucleotide sequence was then analyzed for similarities by using BLASTN tool (*5*).

Production and purification of bacteriocin

The nutrient broth (1000 mL) was seeded with *Bacillus subtilis* R75 10 % (A=1.0 at 540 nm) and incubated at 37 °C and 150 rpm for 72 h. The sample was centrifuged at 10 000×*g* for 20 min and the supernatant was collected. Partial purification of the sample was done by adding $(NH_4)_2SO_4$ at 70 % of saturation level, followed by dialysis for 12 h. The pellet was collected after centrifugation at 20 000×*g* at 4 °C for 30 min. The pellet was dissolved in phosphate buffer (0.1 M, pH=7.0) and stored at 4 °C for further use.

Column chromatography

Sephadex G-100 (5 g) was weighed and suspended in 500 mL of phosphate buffer overnight according to the recommended procedure (*6*). It was swollen for 5 h in boiling water bath followed by deaeration for 1 h and then cooled to room temperature before packing the column. The dimensions of the used column were 75×1.5 cm. The packing was done with a precaution to avoid entrapment of any air bubbles in the gel bed. The column was then washed with phosphate buffer (pH=7.0, 0.1 M). The sample (5 mL) was loaded on Sephadex G-100 column, then it was eluted with phosphate buffer (pH=7.0) and sample fractions (3 mL) were collected in each of total 60 tubes. A flow rate of the sample of 15 mL/h was kept constant. The fractions were analyzed for the protein content by measuring the absorbance at 280 nm and for the bacteriocin activity by using the spot plate method. Bacteriocin positive fractions were pooled together. SDS-PAGE was run to check the purity of the sample as well as to determine molecular mass of the sample (*7*). The purification of bacteriocin was monitored by using 12 % acrylamide gel. Standard molecular mass marker (Sigma-Aldrich, St. Louis, MO, USA) was run along with the sample.

Gel staining

The gel was stained using silver staining method (*8*) and then kept in fixing solution overnight. The next day the gel was put in 30 % (50 mL) ethanol for 30 min and

then kept in Farmer's reagent for 5 min. Three washings of 10 mL each were done with autoclaved distilled water. $AgNO_3$ solution (0.1 %, 100 mL) was added and the gel was put in dark for 30 min. Three washings were done again with distilled water for 20 s each. Developing solution (100 mL) was added and the gel was gently shaken until brown coloured bands appeared in it. The reaction was brought to an end with 1 % acetic acid and the gel was stored in a plastic bag at refrigeration temperature.

Bacillus subtilis R75 activity

The activity of culture supernatant, partially purified and purified bacteriocin produced by *Bacillus subtilis* R75 was calculated by twofold serial dilution (*9*). The culture supernatant, partially purified and purified bacteriocin, was diluted in the range of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10} and 10^{-12}. The smallest detectable zone of inhibition corresponding to the diluted bacteriocin was used to calculate AU/mL.

Characterization of bacteriocin

Effect of pH

A volume of 0.5 mL of purified bacteriocin was added into 4.5 mL of nutrient broth at different pH values ranging from 3.0 to 11.0, with an increment of 1 and incubated for 30 min at 37 °C. Each of the bacteriocin samples treated at different pH values was assayed against indicator bacteria by well diffusion method.

Effect of temperature

A volume of 0.5 mL of purified bacteriocin was added into 4.5 mL of nutrient broth in the test tubes. Each test tube was treated for 10 min at different temperature, *i.e.* 40, 50, 60, 70, 80, 90, 100 and 121 °C after overlaying with paraffin oil to prevent evaporation and the bacteriocin activity was measured by well diffusion assay.

Effect of proteolytic enzyme

Petri dishes containing soft nutrient agar impregnated with indicator bacteria, *viz. L. monocytogenes* and *L. mesenteroides*, were prepared and the effect of proteolytic enzymes on the activity of purified bacteriocin was studied by following the standard method (*10*), where enzyme control 1 (C1) contained 0.3 mL of phosphate buffer, enzyme control 2 (C2) contained 0.15 mL of bacteriocin and 0.15 mL of phosphate buffer, while for the enzyme reaction (ER) 0.25 mg of each trypsin and pepsin (1 mL in 0.5 M phosphate buffer), and partially purified bacteriocin from *Bacillus subtilis* R75 (in a ratio of 1:1) were used.

Effect of stability on the activity of bacteriocin

The activity of purified bacteriocin was checked at regular intervals, *viz*. 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 months. Bacteriocin activity was observed against indicator bacteria, *i.e. L. monocytogenes* and *L. mesenteroides* by well diffusion method, and the size of the inhibition zones was measured. The purified bacteriocin was stored in a clean sterilized glass bottle and frozen (–20 °C).

Mode of action of purified bacteriocin

To determine the mode of action, bacteriocin from *Bacillus subtilis* R75 was mixed with its indicator bacteria in the ratio of 1:1 (1 mL bacteriocin+1 mL of indicator bacteria). The preparation was incubated at 37 °C in 1-hour intervals ranging from 1 to 10 h. The controls (without bacteriocin) were run in parallel. After each time interval, 0.1 mL of the preparation was mounted on the nutrient agar plate by spread plate method. Petri dishes were incubated at 37 °C and colonies of bacteriocin-treated and untreated cells were counted (CFU/mL).

Efficacy of bateriocin

The mixed fruit (orange, *Citrus sinensis*; pineapple, *Ananas comosus* and grapes, *Vitis vinifera* in a ratio 1:1:1) juice (200 mL) was taken in each of the three bottles labelled I, II and III and pasteurized by keeping them in hot water at 72 °C for 2 min. Biopreservative (bacteriocin) and standard chemical preservative (KMS) were added to the juice within the permissible limit, *i.e.* 2000 and 750 ppm to bottle I and bottle II, respectively. The activity of bacteriocin and chemical preservative was studied against the mixed inoculum of *L. monocytogenes* and *L. mesenteroides* (A=1.0, $8.80 \cdot 10^6$ CFU/mL) added to bottles I and II, while bottle III was kept as control, *i.e.* without the addition of any preservative. The samples were stored for 28 days at refrigeration temperature. The morphological changes were also observed in the samples during this period.

Results and Discussion

The strain isolated from dal vari showing hyperproduction of bacteriocin was identified as *Bacillus subtilis* by 16S rRNA gene technique (Fig. 1). It showed 95–96 % homology to the *Bacillus subtilis* used for alignment during BLASTN analysis.

The culture supernatant of *Bacillus subtilis* expressed strong inhibition against many microorganisms/pathogens that cause serious food spoilage, *viz. L. monocytogenes*, *L. plantarum*, *S. aureus*, *B. subtilis*, *C. perfringens*, *B. cereus*, *E. coli* and *L. mesenteroides*, and wide zones of clearance made by crude bacteriocin from *Bacillus subtilis* R75, ranging up to 5 mm, were observed on the Petri dishes

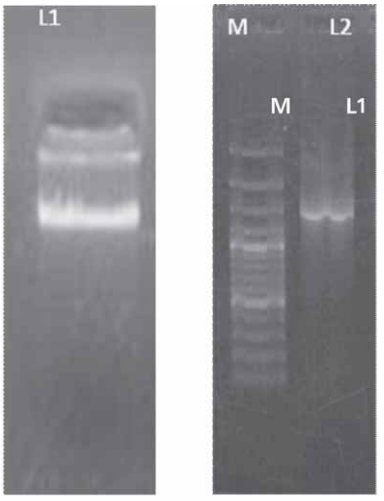

Fig. 1. Molecular characterization of *Bacillus subtilis* R75 using 16S rRNA; L1=genomic DNA, L2=PCR product, M=marker

containing indicator bacteria in well diffusion assay. It showed activity of $10{\cdot}10^5$ AU/mL (Table 1). The strong antagonism against a number of serious and challenging foodborne pathogens/spoilage-causing microorganisms, thus, advocated the high possibility of using this bacteriocin as an effective preservative in food. As cited in literature, bacteriocins are sensitive against closely related species, which limits their application in food (*11*,*12*). However, recent studies have revealed their broad-spectrum activity against a number of microorganisms. A newly isolated bacteriocin from *Bacillus mycoides* can suppress the growth of *L. monocytogenes* and *S. aureus* (*13*). Similarly, paenibacillin (bacteriocin from *Paenibacillus* sp.), was found to be active against many bacteria including *Bacillus* sp., *Clostridium sporogenes*, *Lactobacillus* sp., *Listeria* sp. and *S. aureus* (*14*). This proves that bacteriocins secreted from different bacteria behave differently and have their specific inhibition spectra. Listeriosis caused by *L. monocytogens* in the food items stored even at low temperature in the refrigerator has caused many deaths throughout the world (*15*). Bacteriocin from *Bacillus subtilis* R75 has been found capable of controlling this type of pathogenicity when applied in food, and thus can meet the serious challenge of controlling the spoilage of refrigerated food. Moreover, this bacteriocin is presumed completely safe for human consumption because of its origin from a food grade bacterium.

In order to determine bacteriocin production pattern during growth cycle of *Bacillus subtilis*, the size of inhibition zones against indicator bacteria *L. monocytogenes* and *L. mesenteroides* was measured. The absorbance of the isolate was measured up to 96 h, showing continuous increase in absorbance from 0 to 72 h and a decline afterwards. Bacteriocin production also followed the trend of growth of *Bacillus subtilis.* The inhibition zone size varied from 2 mm for *L. monocytogens* and *L. mesenteroides* at 24 h to 3 and 4 mm, respectively, at 48 h. The maximum production was measured at 72 h with the widest zones formed (5 mm) against both indicator bacteria (Fig. 2). The highest production of bacteriocin by lactic acid bacteria isolated from tempeh was observed during early stationary growth phase (*16*). *Streptococcus macedonicus* ADC-DC198 isolated from Greek kasseri cheese showed bacteriocin production in the early exponential phase, which reached maximum in the stationary phase (*17*).

The crude bacteriocin with a broad spectrum antagonistic activity was further subjected to purification. Partial purification of bacteriocin was achieved by adding $(NH_4)_2SO_4$ to the culture supernatant at 70 % level of saturation. Precipitated bacteriocin caused an increase in

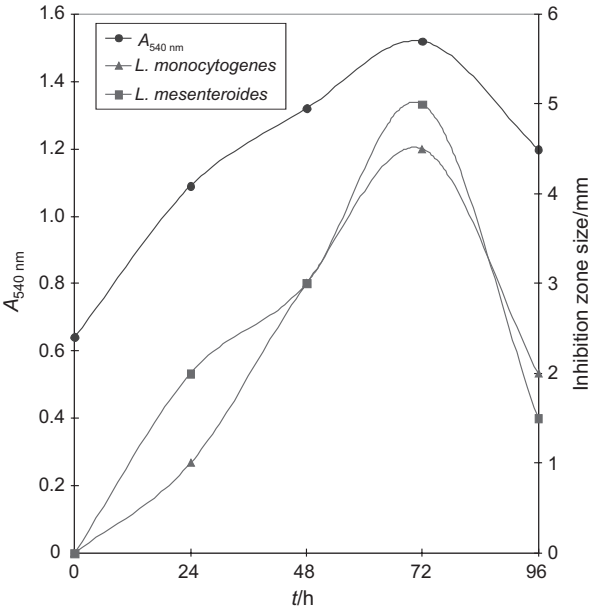

Fig. 2. Production of bacteriocin during growth cycle of *Bacillus subtilis* R75 using 16S rRNA

the size of inhibition zones against the tested strains, indicating stronger antagonistic effect. The partially purified bacteriocin expressed higher activity of $40{\cdot}10^5$ AU/mL (Table 1). Complete purification of bacteriocin from *Bacillus subtilis* was achieved after gel exclusion column chromatography. Fractions 48–52 were pooled together based on their highest antagonistic activity (Fig. 3). Purified bacteriocin produced wider zones of clearance, *i.e.* 12 mm for *L. monocytogenes*, 13 mm for *L. mesenteroides* and 11 mm for *L. plantarum.* The activity of purified bacteriocin increased to $5{\cdot}10^6$ AU/mL (Table 1), *i.e.* its antimicrobial activity against sensitive indicator bacteria thus increased up to 200, 333 and 175 % against *L. monocytogenes*, *L. mesenteroides* and *L. plantarum*, respectively. The increase in the potential of bacteriocin after purification is due to the higher concentration of protein fraction. Homogeneity of the pure bacteriocin sample was proved by a single band obtained after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), as shown in Fig. 4. The molecular mass of bacteriocin was approx. 12 kDa. The single band obtained on the gel was cut and its antimicrobial activity against *L. monocytogenes* and *L. mesenteroides* was recorded, which yielded the inhibition zones of 12 and 14 mm, respectively, thus confirming its status of pure bacteriocin (Fig. 5). Low molecular mass measured in the present study is a characteristic feature of

Table 1. Partial purification and complete purification of bacteriocin produced by *Bacillus subtilis* R75

	V / mL	Activity / AU/mL	Total activity	γ(protein) / mg/mL	Specific activity (10^4)	Purification fold	Recovery / %
Culture supernatant	1000	$10{\cdot}10^5$	$1{\cdot}10^9$	4.651	2.15	1.00	100.00
Partially purified bacteriocin (ammonium sulphate precipitation)	20	$40{\cdot}10^5$	$8{\cdot}10^7$	3.023	13.23	6.09	64.90
Purified bacteriocin (washing+SDS)	10	$5{\cdot}10^6$	$5{\cdot}10^7$	1.031	48.49	22.30	22.10

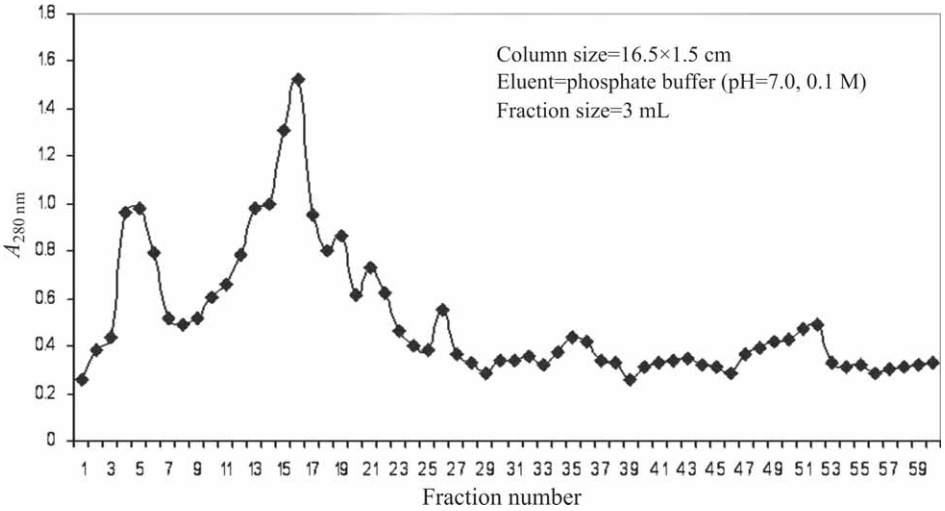

Fig. 3. Elution profile of purified bacteriocin from *Bacillus subtilis* R75 on Sephadex G-100 column

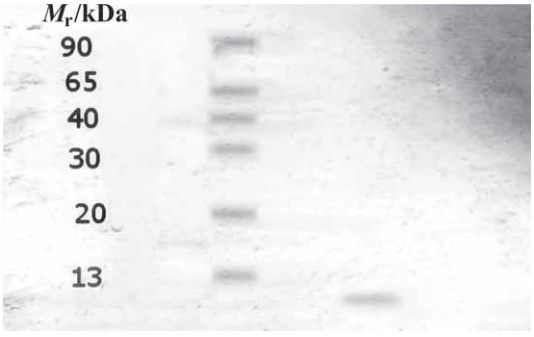

Fig. 4. SDS-polyacrylamide gel electrophoresis of purified bacteriocin from *B. subtilis* R75

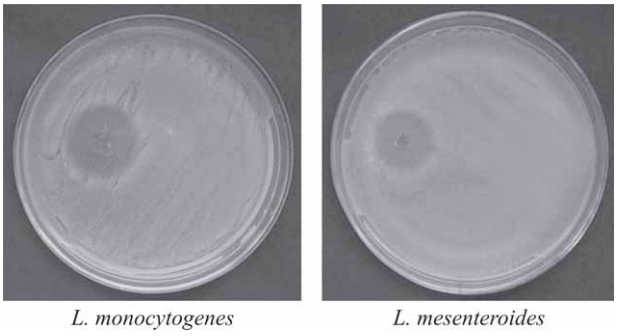

Fig. 5. Inhibitory spectrum of purified bacteriocin against *L. monocytogenes* and *L. mesenteroides* after SDS-PAGE

bacteriocins. Similarly, warnerin 20, a bacteriocin produced by *Staphylococcus warneri* FM20 and purified by SDS-PAGE had a molecular mass of 6 kDa (*18*). A bacteriocin produced by *Lactococcus* sp. GM 005 isolated from miso paste also showed antibacterial activity and had a molecular mass of 9.6 kDa, based on gel filtration analysis (*19*).

The mode of action of purified bacteriocin from *Bacillus subtilis* against test indicator bacteria was evaluated (Fig. 6). When sensitive cells of *L. monocytogenes*, *L. mesenteroides* and *L. plantarum* were treated with purified bacteriocin, the number of viable cells declined from $39{\cdot}10^6$ to $22{\cdot}10^6$ CFU/mL for *L. monocytogenes*, from $34{\cdot}10^6$ to $28{\cdot}10^6$ CFU/mL for *L. mesenteroides* and from $35{\cdot}10^6$ to $19{\cdot}10^6$ CFU/mL for *L. plantarum* in the initial 10 h of incubation. It exhibited bactericidal mode of action clearly. Bacteriocins have generally been reported to dissipate transmembrane potential and increase the membrane perme-

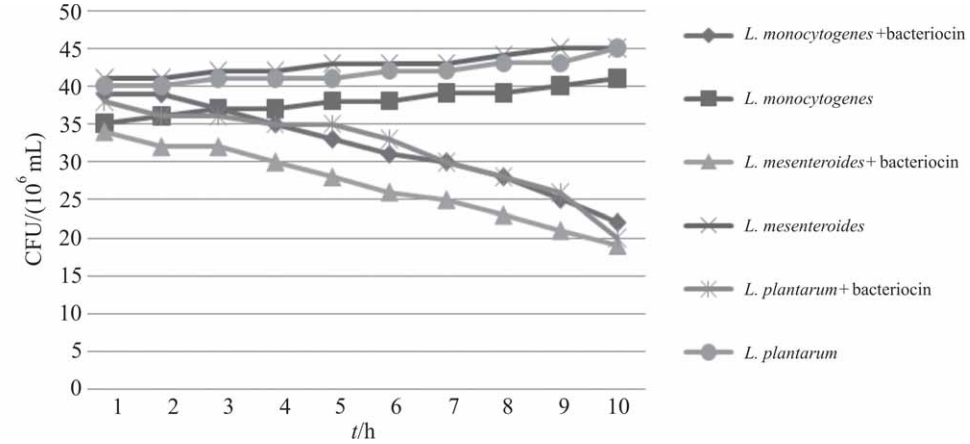

Fig. 6. Bactericidal effect of purified bacteriocin from *Bacillus subtilis* R75 against indicator bacteria

ability to ions, leading to the collapse of the proton motive force and thus killing the cell (*20*). Bactericidal mode of action causes the death of a pathogen, thus, it is capable of eradicating the major population of undesirable microorganisms from food items. It has been observed that warnerin 20 secreted by *Staphylococus warneri* FM20 had bactericidal effect against other sensitive Gram-positive bacteria (*18*).

Pure bacteriocin was characterized in order to assess its capability to work under different environmental conditions from the food preservation point of view. It has been found to be thermostable in nature as it can withstand high temperature, up to 121 °C, although a partial loss in the activity was observed with a continuous increase in temperature (Fig. 7). Thermostability of bacteriocin at high temperature makes it possible to sterilize

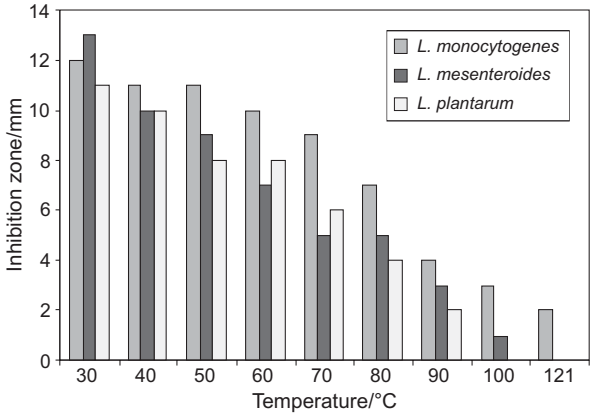

Fig. 7. Effect of temperature on the activity of purified bacteriocin from *Bacillus subtilis* R75

the food products even at room temperature, thus avoiding their storage at refrigeration temperature. The behaviour of bacteriocin towards heat-resistant pathogens also varies. Pediocin produced by *Pediococcus acidilactici* CF2 was found thermostable at 121 °C, while bacteriocin from *Bacillus* strain 8A was found stable up to 80 °C for 30 min (*21*,*22*). On the other hand, thermosensitive bacteriocin from *Brevibacterium linens* was stable at 30 °C only, and its activity was completely lost at 50 °C (*23*). In the present study, purified bacteriocin from *Bacillus subtilis* R75 has worked well in the extended pH range, but the significant activity was observed at pH=6.0 and 7.0 (Fig. 8). Bacteriocin could retain its antimicrobial activity partially when there was a shift to acidic (from 4.0 to 6.0) or basic (from 11.0 to 8.0) range. Stability of bacteriocin at different pH scale is a limiting factor for recommending its use in food items. As reported in literature, bacteriocin from *L. lactis* was found stable in acidic to neutral range (pH=4.0–7.0), while that of *L. plantarum* retained activity from pH=3.0 to 6.0 only (*24*). Lacticin Q secreted by *Lactococcus lactus* was stable in wide range of pH between 3.0 and 10.0 (*25*).

The effect of two proteolytic enzymes, trypsin and pepsin, on the activity of purified bacteriocin was studied. When it was treated with these enzymes in the ratio of 1:1 and welled into Petri dishes containing indicator

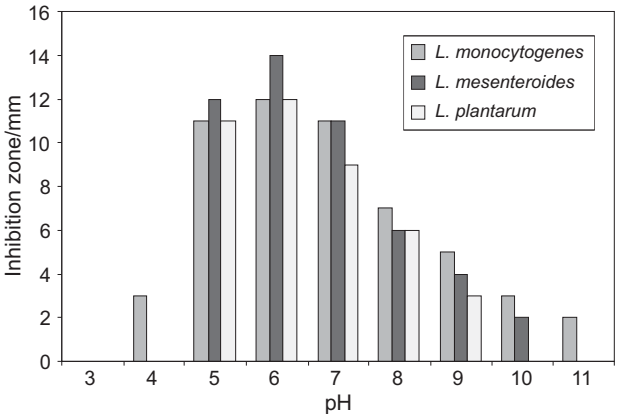

Fig. 8. Effect of pH on the activity of purified bacteriocin from *Bacillus subtilis* R75

bacteria, *i.e. L. monocytogenes* and *L. mesenteroides*, there was either minimum or no inhibition zone formed in the plates, indicating zero activity of bacteriocin (Fig. 9). This proves that bacteriocin is basically a protein in the nature and therefore it can be broken down by gastric juices, thus making it completely safe for human consumption.

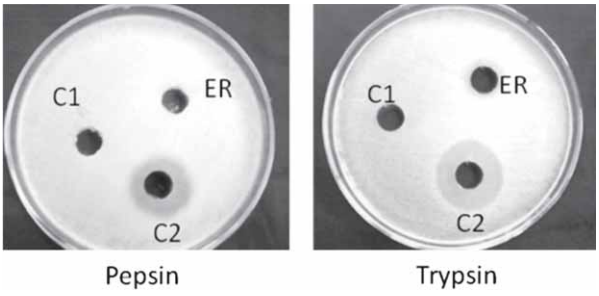

Fig. 9. Effect of proteolytic enzymes pepsin and trypsin on the activity of purified bacteriocin from *Bacillus subtilis* R75 against *L. mesenteroides*; C1=containing 0.3 mL of phosphate buffer; C2= 0.15 mL of bacteriocin and 0.15 mL of phosphate buffer; ER= 0.25 mg of each trypsin and pepsin (1 mL in 0.5 M phosphate buffer)

The purified bacteriocin was stored at freezing temperature for 3 months and its antimicrobial activity was checked periodically at an interval of 15 days. The inhibition zone sizes of 12 and 13 mm were obtained at 15 and 30 days respectively, against *L. monocytogenes* and *L. mesenteroides* respectively, thus retaining full potential up to 1 month of storage time. Thereafter, there was a little decrease in the activity until 2.5 months of storage and the activity became quite low after 3 months of storage (Fig. 10). Therefore, storage of bacteriocin from *Bacillus subtilis* could be recommended for 2.5 months. The inactivation of bacteriocin during storage is probably because when proteins are isolated from their natural congenial environment, they start making new interactions with their surroundings, which is either water or buffer, resulting in the loss of activity (*26*). The storage studies at –20 and –70 °C have shown the loss in the activity of lactococcin R produced by *L. lactis* (*27*) and 50 % reduction in the activity of bacteriocin from *Carnobacterium* sp. after 3 months of storage (*28*).

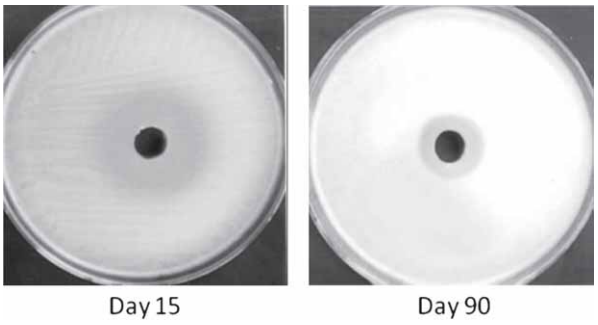

Fig. 10. Effect of purified bacteriocin from *Bacillus subtilis* R75 on *L. mesenteroides* during storage

A comparative study investigating the use of biopreservative and chemical preservative for prolonging the storage of fruit juice inoculated with a mixed culture of *L. monocytogenes* and *S. aureus* was done to test the efficacy of bacteriocin from *Bacillus subtilis* R75. The log CFU/mL of the tested strains declined to 7.87 and 7.00 in the juice containing bacteriocin and KMS, respectively, and reached 9.90 in the control in the 1st week. In the 2nd week, it fell to 6.40 with bacteriocin, while it was 7.10 and 10.60 in the control and with chemical preservative, respectively. The count started changing afterwards, *i.e.* 6.30, 6.40 and 11.90 in the 3rd week and 7.90, 11.50 and 12.70 in the 4th week in juices with bacteriocin, KMS and in control, respectively. The juice in bottle III (control) showed apparent morphological changes, *viz.* change in colour, turbidity, offensive odour and vigorous fermentation. Results have shown statistically that bacteriocin and KMS behaved almost on a par in extending the shelf life of juice. The recommended dose of bacteriocin to be added to food is 5000 ppm (*29*), while only 2000 ppm of bacteriocin was added in this study. The toxicological studies have confirmed that FDA-approved commercialized nisin is not toxic even at a level much higher than used in food production (*30*). Food spoilage can be effectively controlled by bacteriocin because of its activity against pathogens. Among biopreservatives, nisin has already been used successfully at commercial scale in dairy products (*31*). Bacteriocin produced by *Lactobacillus curvatus* was found effective against *L. monocytogenes* in cold smoked salmon during storage at 4 °C, where it lowered the pathogen count up to 22 days (*32*).

Conclusion

Bacteriocin from *B. subtilis* R75 has bright prospects to be used as a food biopreservative as it carries desirable characteristics, *viz.* bacteriocin is secreted from food-grade bacteria already existing in food products, thus rendering them completely safe for consumption, it has strong antagonism against a broad range of serious and challenging foodborne pathogens/spoilage-causing microorganisms, bactericidal mode of action causing death of pathogens, action of bacteriocin at higher temperature and wider pH range, imparting its stability, while degradation of bacteriocin in the presence of proteolytic enzymes makes it completely safe for human consumption. Hence, from the present study it can be concluded that the desirable characteristics of bacteriocin from *B. subtilis* R75 as well as its application to extend the storage life of mixed fruit juice showed high potential of this bacteriocin for use as a natural food preservative in food processing industry.

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