

## Purification of Bacteriocin Produced by *Lactobacillus pentosus* RL2e Isolated from Fermented Cow Milk of Kinnaur Region of Himachal Pradesh

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### Abstract

A bacteriocin producing lactic acid bacterium was isolated from the fermented cow milk of Kinnaur region of Himachal Pradesh and identified as *Lactobacillus pentosus* RL-2e on the basis of morphological, biochemical and 16S rDNA analysis. *L. pentosus* exhibited strong inhibitory potential against food spoilage microorganisms. Maximum bacteriocin production was observed in Elliker broth at 30°C, pH 6.5 and 2% inoculum size. The bacteriocin was purified to 6.9 fold with 3% yield using ammonium sulphate precipitation and gel permeation chromatography. The SDS PAGE analysis revealed that the molecular weight of this bacteriocin was around 20 kDa. The study showed that bacteriocin of *L. pentosus* has good potential as a food preservative.

Keywords: Lactic acid bacteria, bacteriocin, fermented milk, preservative

The biopreservation of foods using lactic acid bacteria (LAB) is catching rapid attention in food preservation. During the last few decades, investigation on food preservation has focused on meeting consumer demands for more natural and healthier foods (Caminiti *et al.*, 2011). This perception, along with the increasing demand for minimally processed foods with long shelf life and convenience, has stimulated further research interest in finding natural and effective preservatives. Lactic acid bacteria provide the major preservative effects in food fermentation which mankind has practised for thousands of years. The primary antimicrobial effect exerted by LAB is the production of lactic acid and consequently reduction of pH (Daeschel, 1989). In addition, LAB produce a variety of antibacterial compounds such as organic acids, diacetyl, hydrogen peroxide, reuterin and bacteriocin during lactic fermentations (Holzapfel *et al.*, 2001; Hirano *et al.*, 2003). Bacteriocinogenic lactic acid bacteria are considered safe additives (GRAS), useful to control the frequent

development of pathogens and spoil age causing microorganisms in foods and feed (Namasivayam *et al.*, 2014). Bacteriocins are antimicrobial proteinaceous compounds with bactericidal or bacteriostatic activity against closely related bacteria to the producer strain i.e. Gram-positive and Gram-negative bacteria (Klaenhammer, 1988; Tagg *et al.*, 1976). The growing consumer demand for finding natural but effective preservation of food, free of potential health risks has encouraged research in the field of biopreservation to find out an attractive and alternative approach to the chemical preservatives.

The most important contribution of these bacteria to fermented products is to preserve the nutritive qualities of the raw material and inhibit the growth of spoilage and pathogenic bacteria (Sadholm *et al.*, 1999). The purpose of this study was to isolate and screen bacteriocin producing LAB, optimize the culture conditions and purify the bacteriocin.

## Materials and Methods

### Chemicals and test organisms

Media ingredients were purchased from Hi Media, India and all the other chemicals were of analytical grade. The test organisms i.e. *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Yersinia pestis* and *Listeria monocytogenes* were procured from Department of Biotechnology, Himachal Pradesh University Shimla.

### Sample collection

The samples of fermented cow milk were collected from different areas of Kinnaur, Himachal Pradesh.

### Isolation and identification of LAB

Lactic acid bacteria (LAB) were isolated from the fermented foods by appropriate dilutions with saline, plated on MRS (de Mann Rogosa Sharpe) agar plate and incubated anaerobically at 37°C for 48 h (Ivanova *et al.*, 2000). After incubation, randomly picked single colonies were transferred to MRS agar plates and incubated at 37°C for 48 h, and were preserved in 40% glycerol at -80°C. Gram positive, non-spore forming and catalase negative isolate was selected for further studies. Further, identification of the species of these *Lactobacilli* was performed according to the carbohydrate fermentation patterns in the MRS broth as described in Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994).

### 16S rDNA gene sequencing of L1 isolate

16S rDNA gene sequencing of L1 isolate was carried out at Xceleris Pvt Ltd Ahmadabad, India. Nucleotide sequence was then analyzed for similarities by using BLASTN tool. Related sequences were obtained from the Gen Bank database. The sequences were aligned by ClustalW and a phylogenetic tree was constructed using Mega Align 6.06 software.

### Screening of isolates for antimicrobial activity

The isolated LAB were screened for antimicrobial activity against indicator organisms, using agar well diffusion method as described by Yang (1992). The indicator organisms were cultured in nutrient broth for 12 h at 37°C and wells were created on the Muller Hinton agar plates and 100 µl of cell free supernatant were filled in 6 mm diameter well. The culture supernatant was adjusted to pH 6.5-7.0 with 1 N NaOH. The plates were incubated for 24 h at 30°C and the diameter of the inhibition zone was measured in millimeters (Harris *et*

*al.*, 1989). Antagonistic activity in arbitrary unit/ml (AU/ml) was calculated as a measure of bacteriocin production.

### Optimization of culture conditions for bacteriocin production

The effect of culture conditions for the maximum production of bacteriocin from isolate L1 isolate was studied. Growth and bacteriocin production were estimated at various temperatures (25°C, 30°C, 35°C, 40°C, 45°C, 50°C and 55°C), pH (3.5, 4.5, 5.5, 6.5, 7.5 and 8.5), inoculum size (2, 4, 6, 8 and 10%) and time (0-48 h). The growth was measured by optical density at 600nm

### Purification of bacteriocin

The bacteriocin was purified using ammonium sulphate precipitation, followed by gel permeation chromatography. Isolate L1 was grown in Elliker broth upto 16 h at 30°C in static condition and the cells were harvested at 10,000 g for 15 minutes. The cell free supernatant of L1 isolate was saturated with 80% ammonium sulphate at 4°C to precipitate out the protein. The precipitated proteins were recovered by centrifugation at 10,000 g for 15 min and suspended in 0.05 M potassium phosphate buffer at 4°C. Further purification was carried out by gel permeation chromatography using Sephadex G-75 column. The dialyzed protein was applied to a Sephadex G-75 column having size of 10 \* 2.5 cm equilibrated with 0.05 M potassium phosphate buffer pH 7. After loading the protein sample, column was washed with potassium buffer to remove the unbound protein and then, eluted with potassium phosphate buffer. The fractions of 1.5 ml were collected and their bacteriocin activity and protein concentration were estimated. The fraction exhibiting maximum activity of bacteriocin was taken for further purification of bacteriocin.

### Determination of protein concentration

Protein concentration of the bacteriocin in supernatant was determined by the dye binding method (Bradford, 1976).

### Molecular weight determination by SDS-PAGE

The molecular weight of the bacteriocin was determined by 15% sodium dodecylsulfate polyacrylamide gel electrophoresis (Laemmli, 1970) and after electrophoresis; the gel was stained with silver staining method. Protein molecular weight markers ranging from 14.3-97.4 kDa were used for the size

determination.

### Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of purified bacteriocin was tested with two fold dilution method. The sample was serially diluted two fold with sterilized phosphate buffer (0.5 mol/ml pH 7.0). One Arbitrary Unit (AU) was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition of the indicator strain (Ko and Ahn, 2000)

## Results and Discussion

### Isolation and identification of bacterial strain

The bacteriocin producing isolate L1 was isolated from the fermented cow milk collected from the Kinnaur region of Himachal Pradesh. The selected isolate L1 was identified as lactic acid bacteria based on its physiological and biochemical characteristics (Table1).

### 16S rDNA gene sequencing and phylogenetic analysis

The bacteriocin producing isolate L1 was identified as *L. pentosus* RL2e by 16S rDNA sequencing. This organism showed 99% homology to *L. pentosus* strain NRIC1557; hence the selected isolate was designated as *L. pentosus* RL2e. Phylogenetic tree constructed using Mega6.06 software showed a close relationship with *L. pentosus* (Fig.1). The sequence was deposited in the GenBank database with accession number KP207686. In earlier studies bacteriocin producing strains have been isolated from various fermented foods i.e. *Lactobacillus rhamnosus* GP1 from grape peel (Sarika *et al.*, 2010), *Lactobacillus plantarum* and *Lactococcus lactis* from fermented vegetables (Joshi *et al.*, 2006) and *Weissella hellenica* D1501 from Chinese Dong fermented meat (Chen *et al.*, 2014)

### Screening of isolates for antimicrobial activity

The antimicrobial activity of *L. pentosus* RL2e was tested against some pathogenic test bacteria i.e. *B. cereus*, *E. coli*, *S. aureus*, *P. aeruginosa*, *L. monocytogenes*, *Y. pestis*, *Shigella* and results are summarized in Table 2. The bacteriocin of cell free supernatant of *L. pentosus* RL2e showed inhibition against pathogens that causes serious food spoilage viz *S. aureus*, *Yersinia*, *E. coli* and *Shigella. dysenteriae* Similarly, *L. acidophilus* strain was reported to be active against *B. subtilis*; *S. aureus*, *E. coli*, *Klebsiella* spp., *S. typhimurium* (Karthikeyan and Santosh, 2009) and *L. rhamnosus* GP1 were reported to be active against *S. aureus*, *P. aeruginosa*, *E. coli*, *B.*

*subtilis*, *E. faecalis* (Sarika *et al.*, 2010). The antibacterial activity might be due to the production of acetic and lactic acid that lowered the pH of the medium or competition for nutrients, or due to production of bacteriocin or antibacterial compounds (Bezkorvainy, 2001; Tambekar *et al.*, 2009).

### Influence of incubation period on the production of bacteriocin

The bacteriocin activity of *L. pentosus* RL2e was maximum in early exponential growth phase (16 h) and activity was measured till 24 h. Maximum bacteriocin production (340 AU/ml) was recorded after 16 h of incubation. In 36 h culture, smaller size of zone of inhibition was observed with respect to decline in OD of cell growth as depicted in Fig. 2

### Effect of temperature, pH and inoculum size on bacteriocin production

The maximum bacteriocin production (360 AU/mL) was observed at 30°C (Fig 3) and pH 6.5 (Fig 4). Significant reduction in the bacteriocin production was observed with the increase in temperature. Among various concentrations of inoculum (2-10% v/v), the high level of bacteriocin production (380 AU/ml) was recorded at 2% inoculum (Fig 5).

Bacteriocin production is strongly dependent on incubation time, pH and temperature (Todorov and Dicks 2004). Similarly, Aslam *et al.* (2012) have reported the highest bacteriocin activity from *Lactococcus lactis* subsp. *lactis* after 18 h, pH 5 and temperature 37°C. The optimum pH and temperature for bacteriocin production by *Lactococcus lactis* CCSULAC1 has been reported as 7.5 and 35°C, respectively (Sharma *et al.* 2010). Therefore it can be concluded that bacteriocin production starts at early log phase and is favored by neutral pH and temperature in the range of 30-37°C.

### Purification of bacteriocin from *L. pentosus* RL2e

The bacteriocin was purified from the cell free extract of *L. pentosus* RL2e using combination of ammonium sulphate precipitation and gel permeation chromatography. The cell free extract was subjected to ammonium sulphate precipitation (0-80%) and the desired protein got precipitated at 0-50% salt saturation. Extracellular bacteriocin was purified up to 1.2 fold with 7.8% yield in ammonium sulphate precipitation (Table 3). The recovered proteins were then fractionated by gel permeation chromatography using 0.05 M potassium phosphate buffer in Sephadex G-75. The fraction 9

exhibited highest antimicrobial activity of 5277 AU/mg and 3.0% yield with 6.9 purified fold were achieved.

Sankar *et al.*, (2012) had reported 13.5 fold bacteriocin purification and yield of 21.3% bacteriocin with specific activity of 1023 AU/mg and molecular weight of 9.5 kDa from *Lactobacillus plantatum*. Another bacteriocin has been purified from *Weissella hellenica* D1501 with 67.52 fold purification, 2% yield and having a molecular mass of 62.42 k Da (Chen *et al.*, 2014).

### Molecular weight determination in SDS-PAGE

Molecular weight of the bacteriocin was determined by SDS-PAGE (Fig. 6). Single protein band was observed when stained with silver stain and it clearly indicated the purity of the protein. The molecular weight of the purified bacteriocin was calculated to be around 20 kDa. Similarly, Wu *et al.*, (2004) purified a bacteriocin from *Pediococcus pentosaceus* ACCEL with molecular mass of 17.5 kDa and categorized it as class IIa bacteriocin. The bacteriocin of lactic acid bacteria belonging to class II and class III have molecular weight <10 k Da and >30 kDa, respectively. Therefore, the bacteriocin of *L. pentosus* RL2e having molecular mass 20 kDa, belonged to class IIa bacteriocin.

### Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of purified

bacteriocin was tested with two fold dilution method. The sample was serially diluted with sterilized phosphate buffer (0.05M, pH 7). Inhibition zone was observed at maximum 1/16 fold of dilution with highest activity of 640 AU/ml against the *Y. pestis*. Therefore, the peculiar antimicrobial characteristics of LAB have very good potential for application as food preservative.

### Conclusion

A bacteriocin was purified from *L. pentosus* RL2e, isolated from fermented cow milk from the Kinnaur region of Himachal Pradesh. The strong antagonistic effect of bacteriocin against the food borne pathogen indicated its potential application in the preservation of food products and use of the isolate *L. pentosus* RL2e as starter culture to improve the hygiene and safety of food products.

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### Conflict of Interest

Authors declare that there is no conflict of Interest.

**Table 1: Physiological and biochemical characteristics of *L. pentosus* RL2e**

Physiological and biochemical characteristics	Observations
Colony morphology	Creamy, smooth round colonies
Gram staining	Gram positive rod, single and paired
Catalase	Negative
Growth in MRS broth	Uniform turbidity
Dextrose , lactose, sucrose and maltose	Fermentation positive

**Table 2: Antimicrobial activity of *L. pentosus* RL2e against pathogenic bacteria**

Pathogenic bacteria	Zone of inhibition (mm)
<i>B.cereus</i>	2
<i>E. coli</i>	7
<i>S. aureus</i>	7
<i>Pseudomonas aerouginosa</i>	—
<i>Listeria monocytogenes</i>	—
<i>Yersinia pestis</i>	5
<i>Shigella dysenterial</i>	4

(—) No. antimicrobial activity.

**Table 3: Purification table for bacteriocin of *L. pentosus* RL2e**

Purification steps	Volume (ml)	Activity (AU/ml)	Total Activity (AU/ml)	Protein conc. (mg/ml)	Total Protein (mg)	Specific Activity (AU/mg)	Purification (Fold)	Yield (%)
Cell free Supernatant	500	380	190000	0.5	250	760	1	100
Ammonium sulphate precipitation	20	750	15000	16	0.8	937.5	1.2	7.8
Gel Permeation	6	950	5700	0.18	1.34	5277.7	6.9	3.0

AU = Arbitrary Unit

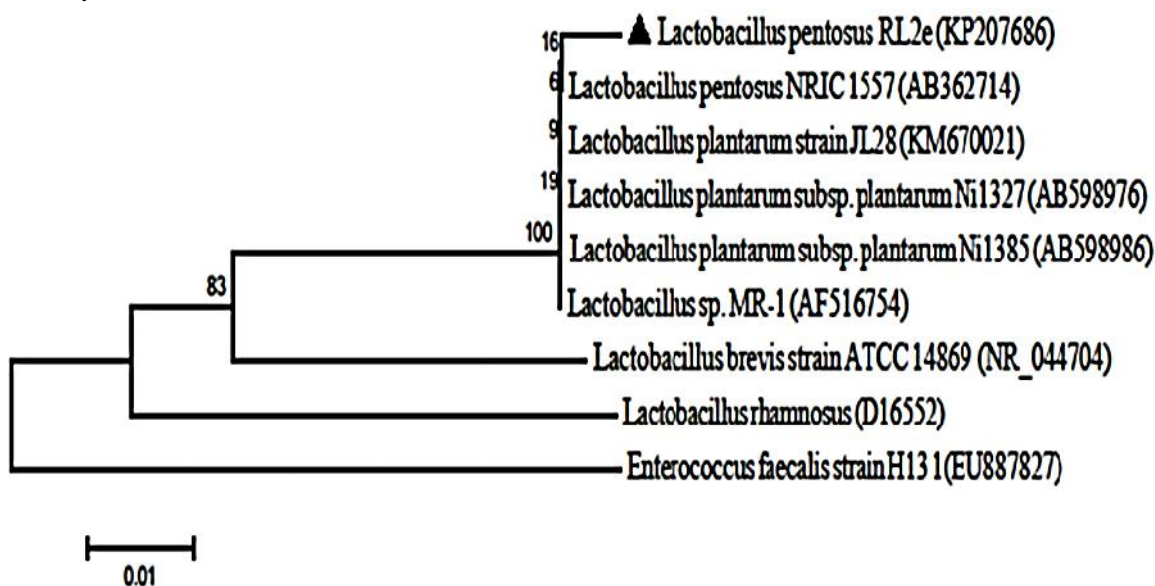


Fig. 1: Phylogenetic tree of *Lactobacillus pentosus* RL2e constructed by Mega 6.06 software, based upon partial 16S rDNA analysis (Accession numbers have been shown in parenthesis). Bootstrap values were based on 1000 replicates and *Enterococcus faecalis* strain H131 was used as outgroup.

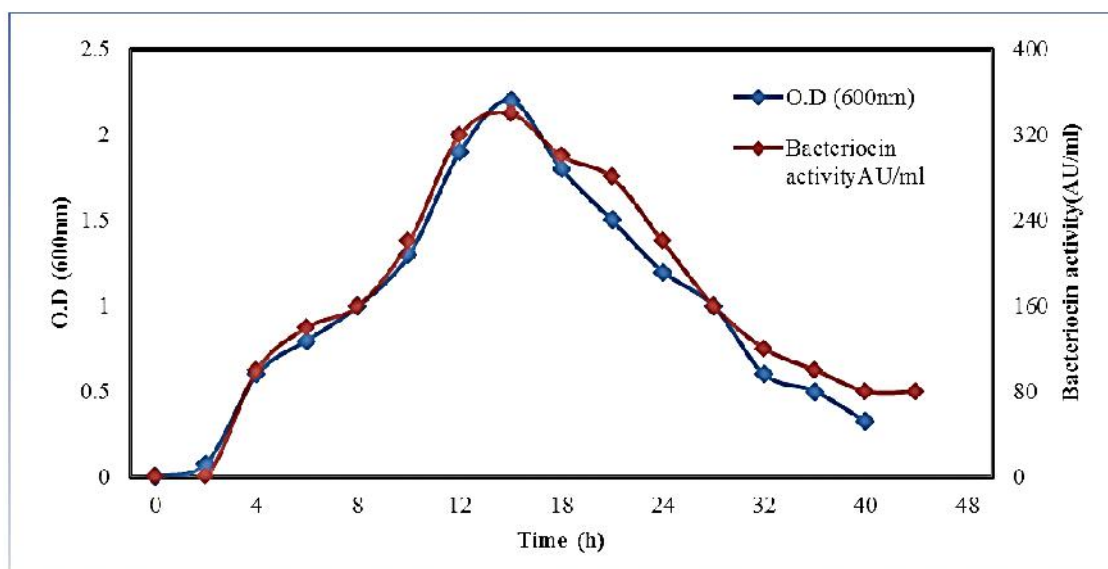


Fig. 2: Growth profile of the *L. pentosus* RL2e and the bacteriocin production

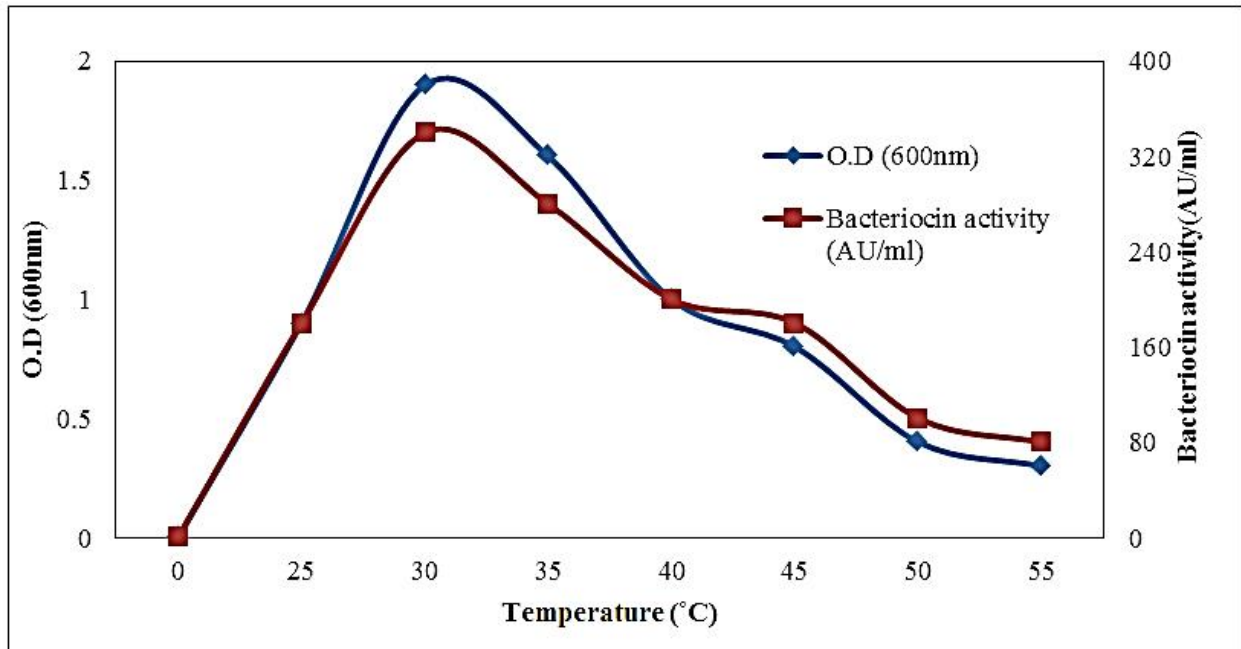


Fig. 3: Effect of temperature on the production of bacteriocin by *L. pentosus* RL2e

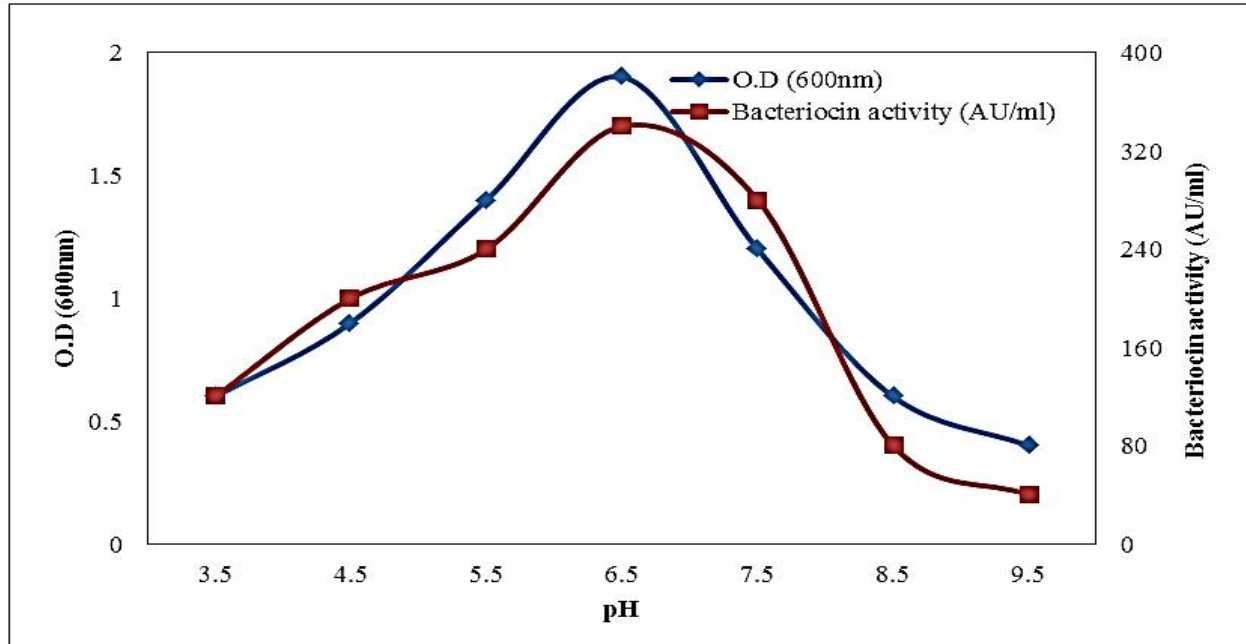


Fig. 4: Effect of pH on growth and production of bacteriocin by *L. pentosus* RL2e

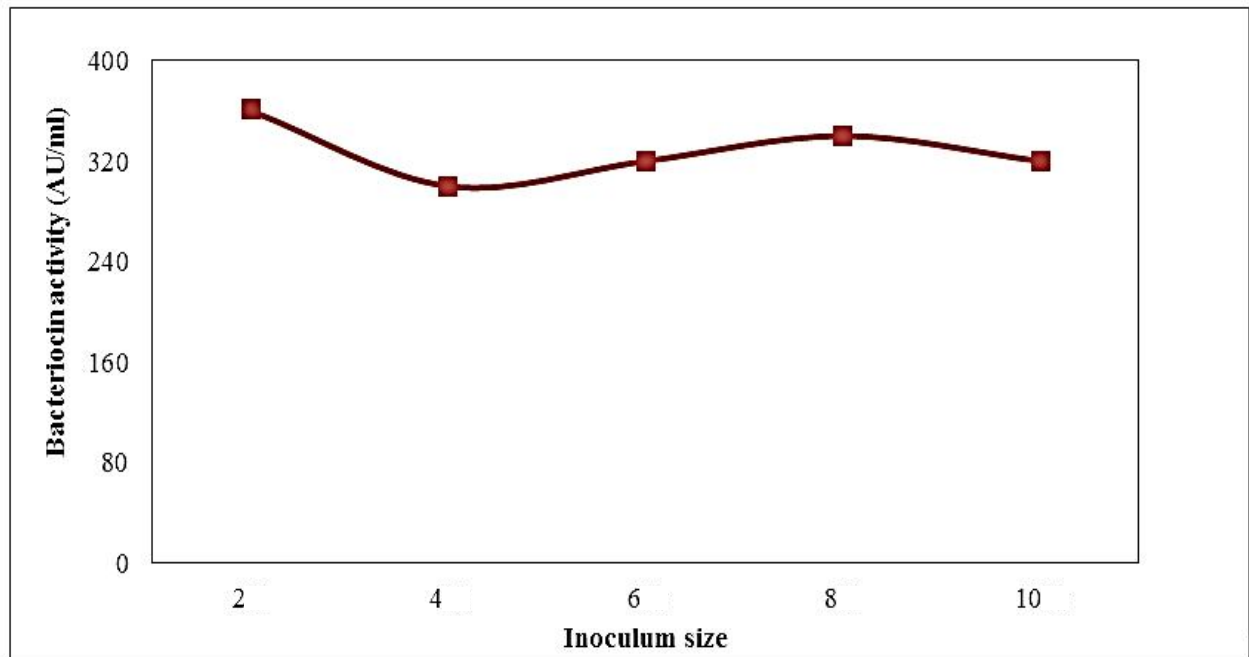


Fig. 5: Effect of inoculum size on the production of bacteriocin by *L. pentosus* RL2e

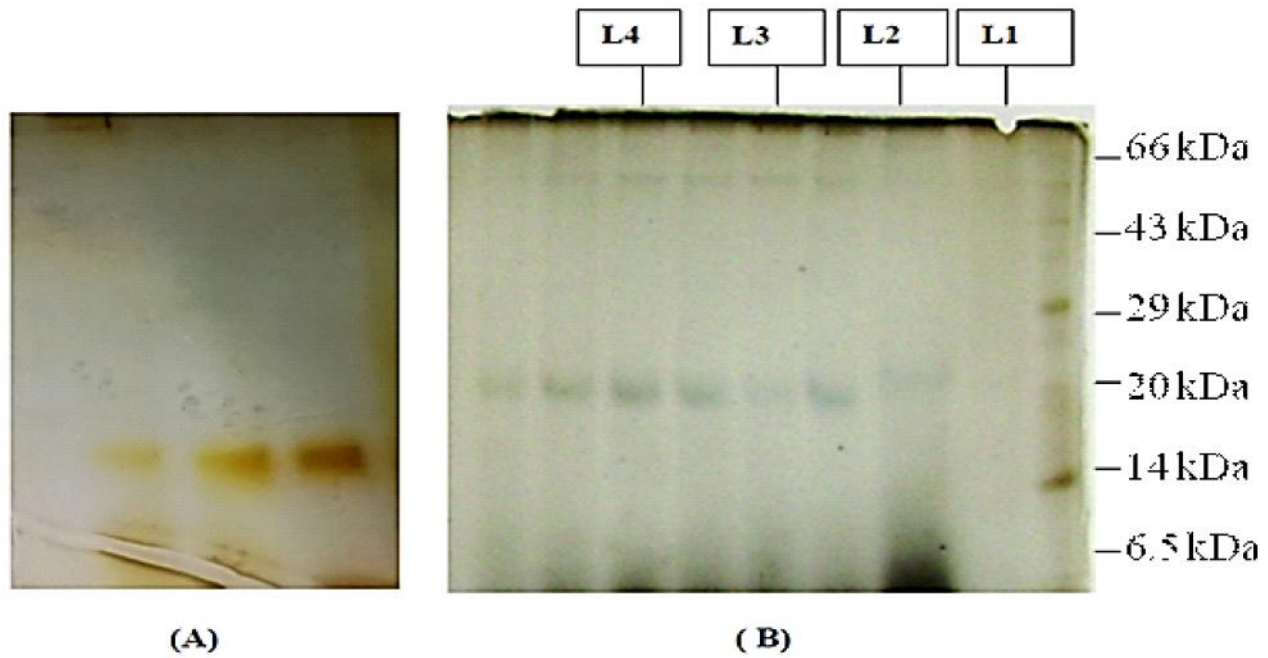


Fig. 6: SDS PAGE analysis of purified bacteriocin visualized by silver staining (A) 10% SDS gel showing purified bacteriocin samples (B) 15% SDS gel where Lane 1: SDS marker L2: Crude enzyme, L3: ammonium sulphate precipitation, L4: dialyzed sample and L4: fractions of gel permeation chromatography having size around 20 kDa

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