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PURIFICATION AND CHARACTERIZATION OF BACTERIOCIN PRODUCED BY *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* R10 ISOLATED FROM FERMENTED RADISH

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ABSTRACT : In the present study, purification and characterization of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* R10, an isolate was obtained from fermented radish was carried out. The bacteriocin produced by *L. lactis* subsp. *lactis* R10 was purified by ammonium sulphate precipitation and cation exchange chromatographic membrane. Ammonium sulphate precipitation resulted in higher yield of bacteriocin, but the specific activity and fold purification were higher for cation exchange chromatographic membrane. The apparent molecular mass of the purified bacteriocin was found to be ~ 3.5 KDa by SDS-PAGE, which revealed single peptide band. Further, the bacteriocin was possessed the thermo-stable property and active at wide range of pH value from 1-10. Amylase, lipase, lysozyme and catalase did not alter the antimicrobial activity but proteolytic enzyme inactivated the bacteriocin. Thus, bacteriocin reported in this study has the potential applications as food preservative agent in vegetable products.

Key words : Bacteriocins, *L. lactis* subsp. *lactis* R10, physical- chemical characteristic- SDS-PAGE.

INTRODUCTION

Lactic acid bacteria (LAB) have been used in industrial manufacture of fermented food products and are known to inhibit the growth of other bacteria by synthesizing a wide variety of low molecular weight antibiotics, metabolic (end) products, enzymes, defective bacteriophages, lytic agents and bacteriocins. Bacteriocins produced by LAB are ribosomally synthesized antimicrobial proteins/peptides and some of these undergo post-translational modifications. Most of the LAB are considered as GRAS (Generally Recognized As Safe) and the use of LAB or their metabolites for food preservation is generally accepted by consumers as natural and health promoting. The use of LAB-generated antimicrobial peptides/bacteriocins as a natural food preservative has gained worldwide attention in recent years because of their inhibitory activity against food spoilage and food-borne pathogenic bacteria such as *Listeria monocytogenes, Bacillus cereus, Clostidium perfringens, Staphylococcus aureus*, *etc*. (Pal *et al*, 2010). The established role of bacteriocin-producing strains in food fermentation and preservation has attracted increasing interest in recent years, either as the components of the natural microflora or raised under controlled conditions (Cintas *et al*, 1998).

Bacteriocins, being biologically derived, low–molecular weight proteinaceous compounds, are considered to be easily degraded during digestion in human beings (Ogunbanwo *et al*, 2003) and, thus, could serve as the best alternative to the predominant method of chemical preservation. Based on their primary structure, molecular mass and heat stability, bacteriocins produced by LAB can be subdivided into four classes. The first class, the lantibiotics, comprises small peptides with dehydrated or modified residues such as dehydroalanine and lanthionine (Klaenhammer, 1993). The second class includes small heat stable bacteriocins such as pediocin A, leucocin A, lactacin F, lactococcins and carnobacteriocin A, BM1 and B2. The third class comprises large heat labile bacteriocins such as helveticin J. The fourth class includes complex proteins that require additional carbohydrates or lipid moieties to attain antimicrobial activity (Jeevaratnam *et al*, 2005).

In an extensive survey of bacteriocin producers, it was observed that about 43% (out of the 162 strains) lactococcal strains tested were capable to produce bacteriocin (Kumari *et al*, 2008). On the other hand, nisin is the only bacteriocin from *Lactococcus lactis* that has been studied in detail. The inhibitory spectra of the different lactococcal bacteriocins vary but they are generally narrower than that of nisin (Geis *et al*, 1983). Nisin occurs as subtypes, A, B, C, D or E, that differ in amino acid composition and biological activity (Hurst, 1981). It is a pentacyclic cationic polypeptide, refered to

as a lantibiotic. Nisin contains 34 amino acids and is synthesized by post-translational processing of ribosomally synthesized precursors (Hansen and Liu, 1990**).** The objective of the present study was to describe a novel bacteriocin produced by *L. lactis* subsp. *lactis* R10 isolated from fermented radish. Further, purification and characterization of bacteriocin was studied in detail for their potential application as food preservative agent in future.

MATERIALS AND METHODS

Bacterial strains, culture media, Growth conditions

The bacteriocinogenic strain *L. lactis* subsp. *lactis* R10 was isolated from fermented radish (*Raphanus sativus)*. The strain was identified by sequencing of 16S rRNA gene followed by blast homology search. The *L. lactis* subsp. *lactis* R10 was grown on MRS broth and the indicator strain *Bacillus subtilis* was grown on Nutrient broth at 37°C for 24 h.

Bacteriocin assay

Bacteriocin activity was determined by using agar well diffusion assay (Tagg and McGiven, 1971). The supernatant of 36 h grown culture was centrifuged at 12, 000 x *g* for 15 min at 4°C, then supernatant was neutralized with sterile 5M NaOH. Aliquots (50 µl) of culture supernatants were applied to disks on agar plates previously inoculated with a cell suspension of *B. subtilis* (10^6 cfu/ml). The plates were incubated for 24 to 48 h and the diameter of inhibition zone around the wells was measured with a calliper. Bacteriocin activity was expressed as Arbitary Units (AU/ml) and defined as the reciprocal of the highest two fold serial dilution showing a distinct zone of inhibition.

Bacteriocin activity (AU/ml) = $2^n \times 100$

n- Highest dilution showing growth inhibition zone

Purification of bacteriocin

Extraction of crude bacteriocin by ammonium sulphate precipitation

The bacteriocinogenic lactic acid bacterial culture was grown in 1L of MRS broth for 36 h at 37^0C. Culture supernatant was collected by centrifugation at 12, 000x *g* for 20 min at 4^0C (Kubota, model - 6800, Japan). The centrifuged culture supernatant was treated with the gradual addition of ammonium sulphate to precipitate the bacteriocin as described below. In 1,000 ml of culture supernatant, ammonium sulphate was added slowly with constant stirring to achieve 40% saturation and the mixture was kept in the refrigerator at 4°C for overnight. Stored mixture was centrifuged at 12,000x *g* for 20 min at 4°C and the collected precipitate (in the centrifuged pellet) was dissolved in sodium phosphate buffer 0.05 M (pH 7.0). The supernatant was subsequently adjusted to 50, 60, 70 and 80% saturation levels by further addition of solid ammonium sulphate. The precipitates in each case were dissolved in sodium phosphate buffer as described above. The surface pellicles and bottom pellets (containing the bacteriocin) were re-suspended in a minimal amount of 0.05 M sodium phosphate buffer (pH 7.0). The individual fraction was separately collected and tested for bacteriocin activity by agar well diffusion assay. The active fractions were pooled together.

Cation exchange chromatographic membrane

The ammonium sulphate precipitated was filtered through 0.45 µm membrane filter, then filtrated was again passed through cation exchange membrane (Acrodisc unit with Mustang S membrane, 0.8 µm, 25 mm, Pall life science, USA) at a flow rate of 1 ml/min. The bound bacteriocin was eluted with linear gradient of (0.2 – 1.0 M) NaCl. Peptides were eluted by increasing the NaCl concentration stepwise using a flow rate of 1 ml/min, resulting in elution fractions with 200 mM (Fraction I), 400 mM (Fraction II), 600 mM (Fraction III), 800 mM (Fraction IV) and 1 M (Fraction V) NaCl. Then individual fraction was tested for bacteriocin activity by agar well diffusion assay. Then pooled and stored at -20ÚC. Protein elution was monitored by measuring the absorbance at 215 nm. The membrane was subsequently washed with NaCl until a stable baseline was reached to remove non-specifically bound material.

Effect of enzyme, heat, pH and surfactants on partially purified bacteriocin activity

Bacteriocin were treated with the following enzymes at a final concentration of 1 mg/ml proteinase K, lipase, á-amylase, lysozyme (Sigma- Aldrich corporation, USA) at 37°C for 2 h and catalase was incubated at 25°C. Untreated samples were used as the control. The thermal stability of the bacteriocin was determined by heating at 40, 60, 80 and 100°C for 20 min and autoclaving for 15 min. The effect of pH was evaluated by adjusting the pH in a range of 2.0-10.0 and incubating at 37°C for 2 h. After heat or enzyme treatment/ pH adjustment, the remaining bacteriocin activity was determined by agar well diffusion assay.

To determine the effect of different surfactant on bacteriocin activity was investigated, by incorporating non ionic (triton X100, tween 20 and tween 80) and anionic (sodium dodecyl sulphate) surfactants. These surfactants were added to partially purified bacteriocin at a concentration of 1%. These preparations were incubated at 37^0C for 1 h and assayed for bacteriocin activity by

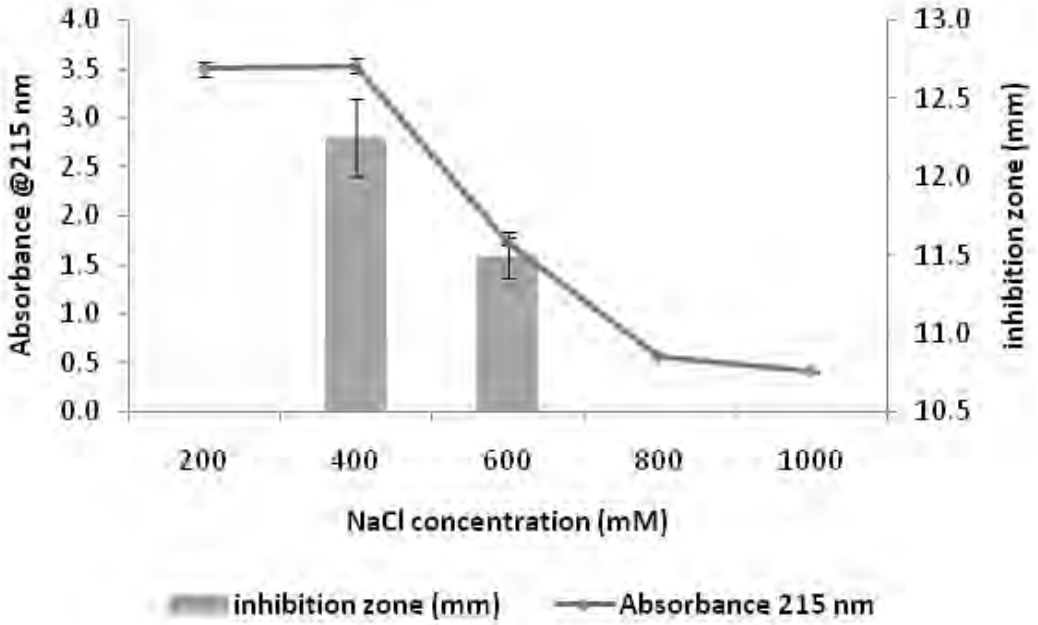

Fig. 1 : Absorbance and inhibition zone of bacteriocin containing fraction eluted at various NaCl concentration.

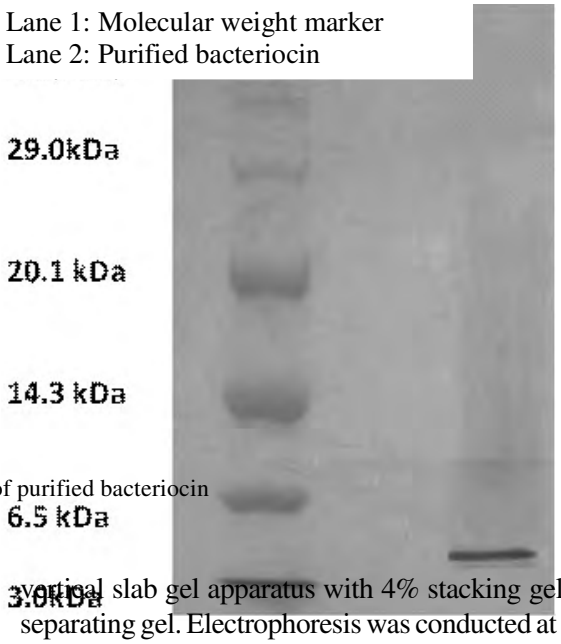

Fig. 2 : SDS-PAGE of purified bacteriocin

agar well diffusion assay.

The treated samples at different temperature, pH and surfactants were tested for their bacteriocin activity against *Bacillus subtilis.*

Molecular weight determination

The molecular mass of the purified bacteriocin of *L. lactis* subsp. *lactis* R10 was determined using sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) as described in Sambrook *et al* (1989) employing a vertical slab gel apparatus with 4% stacking gel and 15% separating gel. Electrophoresis was conducted at a constant voltage of 100 V for 7 h. The molecular weight of the fractionated proteins was compared with standard markers (3.0-43 kDa, Bangalore Genei, India). After completion of run, the gel was stained with coomassie brilliant blue G-250 and destained using methanol: acetic acid solution. The molecular weight of the bacteriocin was calculated from the relative mobility of molecular weight markers run simultaneously.

Table 1 : Purification of the bacteriocin produced by *L. lactis* subsp. *lactis* R10.

Purification level	Volume (ml)	Activity (AU/ml)	Total activity (AU)	Protein (mg/ml)	Total protein (mg)	Specific activity (AU/mg)	Recovery (%)	Purification (fold)
Cell free supernatant	1000	644	644000	2.17	2170	296.7	100	1.0
Ammonium sulphate precipitation	20	3200	64000	1.2	24	2666.7	9.9	8.98
Cation exchange chromatography	10	1500	15000	0.37	3.7	4054.0	2.3	13.6

Table 2 : Effect of enzymes, temperature and pH treatment on bacteriocin activity.

Application	Activity (AU/ml)
Enzymes	
Control	3200
Proteinase K	-
α – amylase	3200
Lipase	3200
Catalase	3200
Lyzosyme	3200
Temperature	
40^{0}C for 20 min.	3200
60^{0}C for 20 min.	3200
80^{0}C for 20 min.	3040
100^{0}C for 20 min.	2971
121^{0}C for 15 min.	2665
pH	
2	3200
4	3200
6	3200
8	2745
10	2171

Table 3 : Effect of surfactant treatment on bacteriocin of *L. lactis* subsp. *lactis* R10.

Application	Concentration (%)	Bacteriocin activity (AU/ml)
Control	-	3200
Surfactants		
Tween 80	1.0	3200
Tween 20	1.0	3200
TritonX-100	1.0	3300
SDS	1.0	3200

Protein quantification

The amount of protein in various samples was determined by the method of Lowry *et al* (1951) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The bacteriocin was secreted into the growth medium and maximum bacteriocin activity was obtained from the cultures in the stationary phase. This bacteriocin was purified to apparent homogeneity using a two-step purification protocol. The bacteriocin was pre-purified by ammonium sulphate precipitation. Different saturation levels of ammonium sulphate such as 20%, 40%, 50%, 60% and 70% were done to precipitate the bacteriocin. The bacteriocin activity was observed up to 40% ammonium sulphate saturation and thereafter no bacteriocin activity was observed. At this step, an almost nine-fold increase in specific activity was recorded. The recovery was approximately 10%.

The ammonium sulphate precipitate was passed through the cation exchange chromatographic membrane using the five step NaCl elution gradient. When this sample was applied on cation exchange chromatographic membrane, the sample was collected at one ml per minute and it was collected at five different fractions. Among the five fractions, the bacteriocin activity was found only in fraction II and III of the eluted samples (Fig.1). Analysis of absorbance at 215 nm revealed that, a high absorbance at 215 nm occurred at the first elution fraction, but this did not correspond to bacteriocin as evidenced by agar well diffusion assay. The elution fraction II and III contains large amount of bacteriocin as evidenced by agar well diffusion assay. The pooled protein fractions revealed the bacteriocin activity, which yielded (at recovery of 2.3% and 13.6 fold purification as indicated in Table 1) a titre of 1500.00 AU/ml and a specific activity of 4054.0 AU/mg protein. In this study, low recovery rate of bacteriocin were observed. Notably, other researcher were also obtained low recovery rates for lactacin B (2.4% recovery) (Apolonia *et al*, 2008) and acidocin 8912, (13.6% recovery) (Tahara *et al*, 1992). On the other hand, high recovery rate (41% recovery, 369-fold purification) was obtained for lactacin F (Cameilla *et al*, 2008).

All the antimicrobial substances were inactivated by proteinase K but not affected by treatment with á-amylase, catalase, lysozyme and lipase (Table 2). Treatment with amylase did not change the antimicrobial activity of the bacteriocin, suggesting that the bacteriocin

was not glycosylated. Antimicrobial activity of the bacteriocin was unaffected by treatment with catalase (Albano *et al*, 2007). The active principle of bacteriocin produced by *L. plantarum* F1 was proteinaceous in nature, since the bacteriocin was inactivated by proteolytic enzymes (Pal *et al*, 2010). Protease sensitivity is a key criterion for the characterization of bacteriocin, as it confirms its proteinaceous nature.

The effects of heat on bacteriocin activity were determined by heating partially purified samples of bacteriocin at various temperatures (40, 60, 80, and 100 for 20 minutes and 121°C for 15 min.) using *B. subtilis* as indicator organism. The bacteriocin activity was unaffected by heating at moderated temperatures and at 100°C for 20 min. The bacteriocin produced by the isolates was considered to be most heat stable, as the activity (2665 AU/ml) remained after heating at 121ÚC for 15 min. at 15 psi pressure. Compared with unheated partially purified, bacteriocin activity was reduced by 7.2% and 16.19% for treatment at 100°C for 20min and 121°C for 15 min respectively. This study clearly demonstrated that the bacteriocin obtained from strain R10 is thermostable (Table 2). The reason for the bacteriocins heat stablility could be due to its complex nature. Several studies have been reported that the bacteriocin treated at 100°C for 120 min and 121°C for 15 min were stable at this high temperature (Pilar *et al,* 2008). Pediocin SJ-1 (Schved *et al*, 1993) was not affected by heat treatment for 30 min at 100°C. Likewise, Vandenbergh *et al*. (1993) showed that nisin produced by eight *L. lactis* strains isolated from vegetables did not loose their activities after treatment at 100°C for 2h. These examples clearly indicates that bacteriocin possess thermostable property. The heat stability of bacteriocin discussed here indicates that it could be used as biopreservative in combination with thermal processing to preserve the food products. Furthermore, when comparatively low temperature is employed for processing compared to high temperature being used at present, the retention of nutrients would be higher.

The effect of pH on activity of bacteriocin was carried out. It was observed that bacteriocin produced by *L. lactis* subsp. *lactis* R10 was active in wide range of pH from 2-10 Table 2. The bacteriocin activity was stable (3200 AU/ml) for *B. subtilis*, when the pH of the partially purified bacteriocin was between 2.0 to 6.0. Bacteriocin activity against *B. subtilis* was lost 14.22% and 32.15% of its activity at pH 8.0 and 10.0 respectively. Nisin is the most stable at pH 2.0 and its activity decrease drastically or is lost at pH>7.0 at room temperature (Hurst, 1981). Kumari *et al* (2012) reported that bacteriocin produced by *L. lactis* subsp. *lactis* LL171 was active at wide range of pH value from 1-11. Bacteriocin from *L. lactis* subsp. *lactis* R10 was not only active and stable over a wide pH range but it was also extremely heat stable at neutral pH values, indicating that it can be useful in acidic and non-acidic foods. The stability of bacteriocin to different conditions reflects that such compounds can withstand the conditions normally encountered in food processing, so would remain effective during processing.

Exposure to Triton X-100 resulted in an increase in the bacteriocin activity 3300 (AU ml^{-1}) for *B. subtilis* respectively where as tween 20, tween 80 and SDS maintained the bacteriocin activity. The exposure of the bacteriocin samples to surfactants resulted in an increase in the bacteriocin titre. This increase might be due to the effect of surfactant on the permeability of the cell membrane (Graciela *et al*, 1995). The increase in bacteriocin activity could be due to attributable to dispersion of the bacteriocin complex thereby releasing more units for the activity (Diop *et al*. 2007).

The molecular weight of the purified bacteriocin was found to be 3.5 kDa as judged by SDS-PAGE (Fig. 2). Previous studies showed that the molecular weight of nisin extracted from growth media was about 3.5 KDa (Gross and Morell, 1967; Jarvis *et al*, 1968; Gross, 1977). Zendo *et al* (2003) reported the nisin Q molecular weight 3.4 KDa.

CONCLUSION

Our results confirmed that all inhibitory substances produced by *L. lactic* subsp. *lactis* R10 strain were bacteriocins. The sensitivity of these bacteriocins to proteolytic enzymes indicated their proteinaceous nature. Similar to nisin in molecular weight, the bacteriocin of strain R10 was extremely thermostable and pH stable. Therefore, it has a potential for application as a biopreservative in different thermally processed food products as such or in combination with other preservation methods.

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