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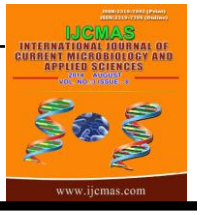

Original Research Article

Characterization and antimicrobial properties of partially purified pediocin produced by *Pediococcus acidilactici*

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Keywords

Pediococcus; Bacteriocin; Antibacterial: Purification, Biopreservative

A B S T R A C T

Pediococcus acidilactici isolated from fermented milk product (curd) produced a bacteriocin showing broad spectrum antimicrobial activity which was active against many food pathogens and spoilage microorganisms. The bacteriocin was extracted by cell adsorption-desorption method and purified through Sephadex G-25 column. The purity of the sample was confirmed by reverse phase high pressure liquid chromatography (RP-HPLC). The purified bacteriocin named as pediocin was found to be a protein entity through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with an approximate molecular weight of 5.5 kDa. The bacteriocin was sensitive to proteolytic enzymes and stable over wide range of temperature (70 to 90^{o}C) and pH (4.0 -7.0).The broad spectrum of its antimicrobial activity and stability indicated its potential application as biopreservative in processed and non-fermented foods.

Introduction

Lactic acid bacteria (LAB) are industrially important organisms because of their fermentative ability as well as health and nutritional benefits. Moreover they are generally regarded as safe (GRAS) for incorporation into food products and are of major interest in the food industry especially in the preparation of dairy products, meat products and wine. Earlier researchers have isolated LAB from dairy and meat products, fermented vegetables and mucosal surface of animals (Jamuna et al, 2004; Mahantesh et al, 2006-2007; Mahantesh et al, 2010) and were shown to be associated with antagonistic property of inhibiting the growth of food spoilage and pathogenic microorganisms. This property of LAB in food products is due to competitions for nutrients and the presence of starter derived inhibitors such as lactic acid, hydrogen peroxide and bacteriocins (Lindgren and Dobrogosz, 1990; Cleveland et al, 2001).

Bacteriocin producing species have now been identified among all the genera of LAB including *Lactobacillus*, *Pediococcus* and *Carnobacterium* as well as several *Enterococci* and *Weissella* organisms. Bacteriocins are ribosomally synthesized extracellularly released low molecular mass

peptides or proteins (usually 30-60 amino acids) which exhibit either bactericidal or bacteriostatic effect on other bacteria either in the same species or across the genera. Bacteriocins of LAB are considered as safe and natural food biopreservatives as they are degraded by proteases in the gastrointestinal tract (Kim et al, 2000). The inhibitory spectrum of bacteriocins also includes food spoilage and/or food-borne-pathogenic microorganisms. Earlier we have isolated a bacteriocin producing *Pediococcus acidilactici* from fermented dairy products (Mahantesh et al, 2010) and in this study an attempt was made to purify and characterize the bacteriocin by this strain.

Several strains of *Pediococcus acidilactici* and *Pediococcus pentosaceous* were found to produce pediocin (Kim et al, 2000; Jamuna et al, 2004; Vijai et al. 2010). Most of them are small heat-stable and non-lanthionine containing peptides belonging to the class-II bacteriocins (Klaenhammer, 1988). Pediocin has been shown to be more effective than nisin against some food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus.* However, inspite of its potential application as a food biopreservative, the pediocin has been neither legally approved by the regulatory agencies nor available commercially. *Pediococcus acidilactici* (Accession No FJ390108) isolated from fermented dairy product (curd) from India was found to produce bacteriocin active against some food pathogens and spoilage bacteria. The present paper describes the extraction, purification and characterization pediocin produced by *Pediococcus acidilactici* .

Materials and Methods

Bacterial strains and chemicals

Pediococcus acidilactici bacteriocin producer strain was isolated from curd and identified by sequencing its 16S rRNA gene followed by BLAST homology search. The nucleotide sequence was deposited with NCBI database under accession number FJ390108. The indicator organisms used in the study were procured from Microbial Type Culture Collection (MTCC) Chandigarh, India. All the chemicals used in the study were procured from Sisco Research Laboratory (SRL) India, while molecular weight markers, enzymes and bacteriological media were obtained from Sigma, USA and HiMedia Pvt Ltd, India, respectively.

Extraction and partial purification of bacteriocin

Extraction by cell adsorption-desorption method

Pediococcus acidilactici was grown at 37^{o}C for 48 h in de Mann Rogosa Sharpe (MRS) broth and the bacteriocin was extracted by cell adsorption-desorption method (Yang et al, 1992) with minor modifications. After the growth, culture media of the isolate was heated at 80^{o}C for 20 min to inactivate proteases and later adjusted to pH 6.5 and made bacteriocin to adsorb to cells by slow stirring at room temperature (25-30^{o}C) for 4 h. Later the cells were pelleted and washed with 5 mM sodium phosphate buffer of pH 6.5 and bacteriocin was desorbed into 0.1N NaCl pH 2.5 by stirring overnight under cold conditions (6-8^{o}C).

Cells were separated by centrifugation and supernatant was adjusted to pH 5.0 and concentrated by rotary flash evaporator and further purified by gel permeation chromatography (GPC). This bacteriocin was used to study the biochemical properties and determine the molecular weight of bacteriocin by Tris-Tricine SDS-PAGE as described below.

Gel Permeation Chromatography and RP-HPLC

A column (70 X 2 cm) was packed with Sephadex G-25 powder swollen in distilled water for 48 h, equilibrated in ammonium acetate buffer (0.05 M, pH 4.8) and the same buffer was used to elute the sample. Void volume was determined by passing 2.0 mg/ml blue dextran (2000 kDa) through the column. The bacteriocin sample obtained by cell adsorption-desorption method was passed through the column. Fractions were collected after passing of void-volume at a flow rate of 0.4 ml/min at 4 min interval and optical density (OD) of the fractions was measured at 280 nm. Various fractions around the shoulder, peak and valley were pooled separately and were checked for antimicrobial activity against various food pathogens by agar well diffusion method (Fig. 1).

Purity of the active fractions was tested by injecting 20 µl of sample into an analytical RP-HPLC (Waters 600 analytical HPLC system, Milford. MA, USA) equipped with analytical column (C_{18}, 4.6mm x 250 mm, pore size 80 Å). The buffer A consisted of 0.1% trifloroacetic acid (TFA) in water and the buffer B consisted of 0.1% TFA in acetonitrile. Fourty five min linear gradient from 100% A/ 0% B - 70% A/ 30% B at flow rate of 0.5 ml/min was used for sample elution which was monitored in the range 210-400 nm.

Protein assay

Protein concentration of purified bacteriocin was determined by modified Biuret method (Gornall et al, 1949) with bovine serum albumin as standard.

Tris-Tricine SDS-PAGE analysis

Tris-tricine SDS-PAGE was employed to determine the molecular weight of purified bacteriocin sample (Schagger et al, 1987). A vertical slab gel electrophoresis apparatus with 16% separating gel, 10% spacer gel and 6% stacking gel of acrylamide-bis acrylamide was used. The molecular weight of bacteriocin was calculated from the relative mobility of the standard molecular weight markers (Sigma) run simultaneously at 60 V for 12 h.

Antimicrobial activity assay

Antimicrobial activity of the bacteriocin against food pathogens and spoilage bacteria shown in the Table 1 was studied by agar well diffusion method (Tagg and McGiven, 1971) with minor modifications and the results were expressed in terms of zone of inhibition and AU/ml of bacteriocin (Kim et al.. 2000). Seventy five microliter of bacteriocin was loaded into the wells of Tryptone Soya agar (0.8%) plates spreaded with an overnight culture of test organism at a concentration of 10^7-10^8 cfu/ml. The plates were incubated at 37^{o}C for 18 h and were examined for zone of inhibition.

Characterization of bacteriocin
Effect of temperature

An aliquot of partially purified bacteriocin was heated at 70^o, 80^o, 90^o, 100^{o}C for 30 min and 121^{o}C for 15 min and then assayed for antimicrobial activity against *Staphylococcus aureus* along with the untreated sample (control).

Effect of pH

The effect of pH on bacteriocin was tested by adjusting pH of the bacteriocin for 24 h in the range of pH 3.0 to 9.0 and incubated at room temperature. The pH adjusted samples were incubated at room temperature for 24 h and then neutralized to pH 5.0 and tested for its residual antimicrobial activity.

Effect of enzymes

Tolerance of bacteriocins to various enzymes such as trypsin, protease (3 mg/ml) lipase, amylase and catalase 2 mg/ml was studied by incubating at 37^{o}C for 1 h. After incubation the enzymes were inactivated by heat treatment at 65^{o}C for 30 min and tested for residual bacteriocin activity. Controls were maintained without any treatment of bacteriocin.

Results and Discussion

Extraction and partial purification of bacteriocin

The crude bacteriocin obtained by cell adsorption-desorption technique was purified by gel permeation chromatography and the fractions were collected after elution of void volume. Among the fractions collected, the fractions 13-26 were found to show antimicrobial activity against *Staphylococcus aureus* (Fig. 1). These active fractions were pooled and concentrated to 1.0 ml and used for purity check by RP-HPLC. A single peak was observed in the range 210-400 nm, which was later extracted at 280 nm (Fig 2) indicating the purity and homogeneity of the bacteriocin sample.

SDS-PAGE analysis of bacteriocin sample

Purified bacteriocin from active fractions was analyzed by Tris-Tricine SDS-PAGE and showed a single protein band. The molecular weight of bacteriocin as determined by relative mobility was approximately 5.5 kDa (Fig 3). The molecular size of this protein is in agreement with earlier reports (Kim et al, 2000; Jamuna et al, 2004; Vijai et al, 2010). This is also a confirmation that the pediocin is pure and indicates that the bacteriocin is a relatively small peptide as are weisselicin 110 (Srionnual et al, 2007) and lactocin F (Bhunia et al, 1988).

Antimicrobial spectrum

Pediocin showed its antimicrobial activity against many food-borne pathogens and spoilage organisms including Gram negative bacteria which are normally resistant to bacteriocins (Table 1). The antimicrobial activity was observed maximum against Gram positive bacteria when compared to Gram negative organisms.

Characterization of bacteriocin
Effect of temperature, pH and enzymes

There was no significant change in inhibitory activity of bacteriocin after 30 min of heating at 70^{o}, 80^{o} and 90^{o}C and pH 4.0 to 7.0. There was 82 and 70% of its residual activity left even after 30 min at 100^{o}C and 15 min at 121^{o}C heating and 62% and 65% of residual activity at pH 3 and 8. These data showed that the bacteriocin was heat stable and could retain its activity over a wide range of pH (Table 2).

Treatment of bacteriocin with protease and trypsin completely inactivated the bacteriocin but bacteriocin treated with amylase, lysozyme and catalase did not affect its antimicrobial activity suggesting the purified bacteriocin is a protein type (Table 2). Lactic acid bacteria are endowed with ability to produce several antimicrobial compounds including bacteriocins that varied in their spectrum of activity. In the present study pediocin from *Pediococcus acidilactici* was purified by GPC and some of its characters were evaluated. In addition a simple medium was designed for its bulk production and purification. Pediocin showed wide range of strong antimicrobial activity against many Gram positive and Gram negative bacteria.

Fig.1 Elution profile of pediocin on Sephadex G-25 column.
Active fractions indicated by line

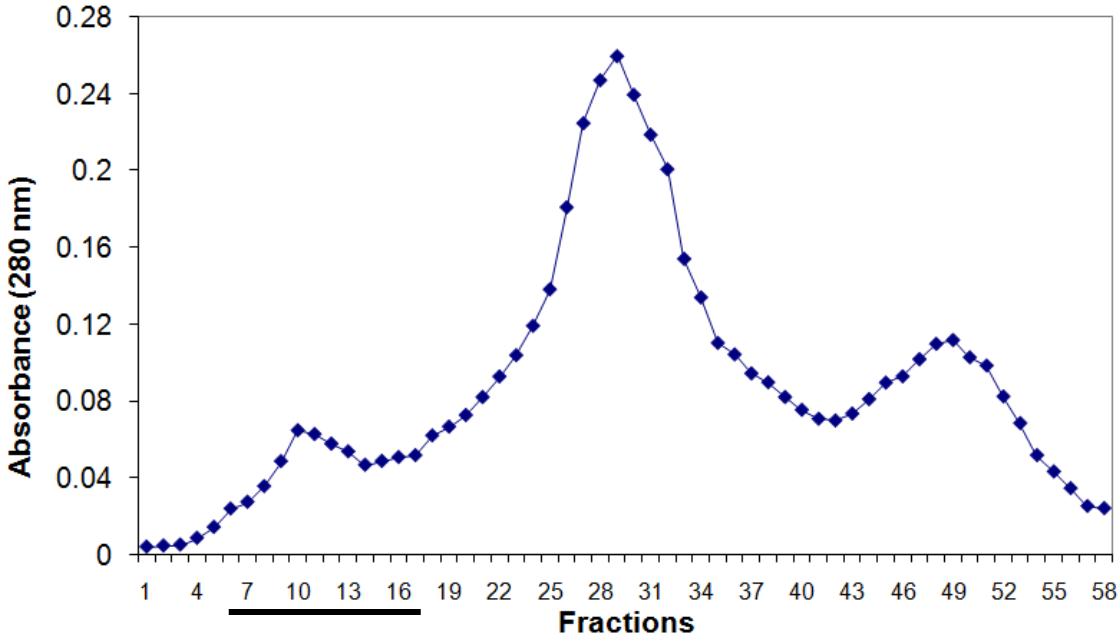

Fig.2 Elution profile of pediocin in RP-HPLC (peak retention time 3.30 min)

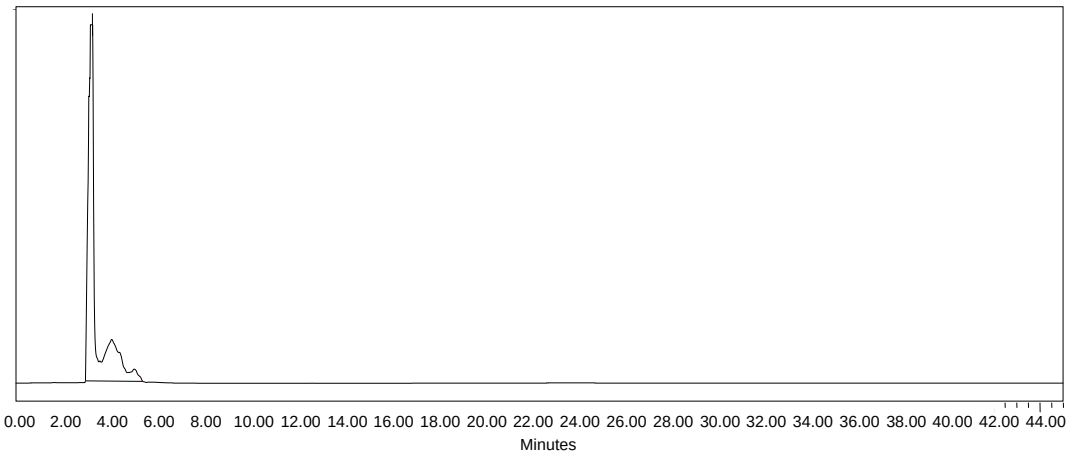

Fig.3 Polyacrylamide gel electrophoresis of purified pediocin
Lane 1, Molecular weight standard (Sigma),
Lane 2, Purified pediocin (arrow indicates location of pediocin).

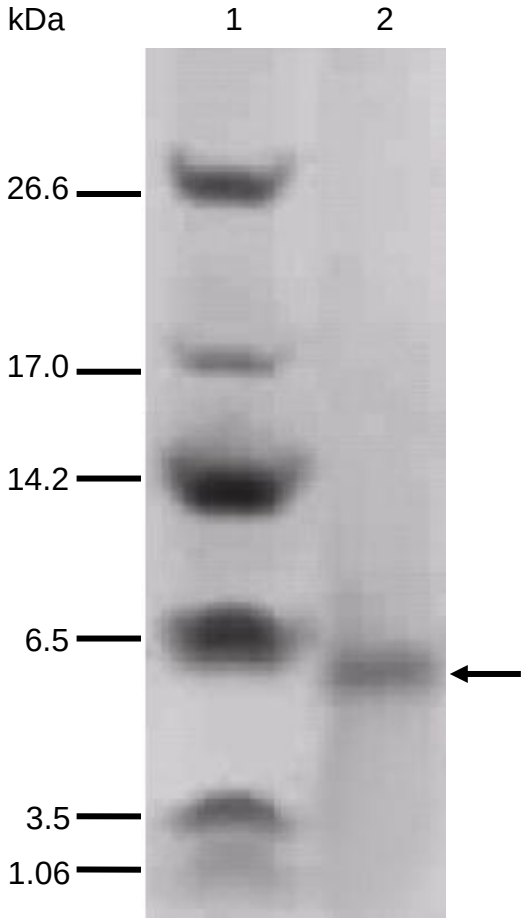

Table.1 Antibacterial spectrum of pediocin by agar well diffusion method

Indicator organisms	Zone of inhibition (mm)[a]	AU/ml of bacteriocin
Staphylococcus aureus MTCC 737	15 (14-16)	1780 (1530-2010)
Micrococcus luteus MTCC 2452	13(12-15)	1650 (1435-1870)
Listeria monocytogenes MTCC 657	11 (9-13)	1390 (1150-1560)
Bacillus cereus MTCC 1272	10 (8-12)	1185 (1075-1265)
Bacillus subtlis MTCC 441	9 (7-11)	1105 (975- 1250)
Yersinia enterocolitica	10 (8-12)	1160 (1105-1210)
Aeromonas hydrophilus MTCC 646	9 (8-10)	1145 (1010-1350)
Escherichia coli DFR 262	10 (9-11)	1110 (1070-1200)
Pseudomonas DFR 219	13 (10-15)	1455 (1250-1155)
Vibrio parahaemolyticus MTCC 451	10 (8-12)	1080 (950-1155)
Salmonella typhimurium MTCC 98	10 (9-11)	1050 (930-1180)

[a] The values are means of 3 independent experiments performed in duplicates; the range is given in parenthesis.

Table.2 Effect of temperature, pH and hydrolytic enzymes on the stability of pediocin

Treatment		Residual bacteriocin activity (%)[a]
Temperature ^{o}C	70 for 30 min	100
	80 for 30 min	100
	90 for 30 min	91 (86-96)
	100 for 30 min	82 (75-89)
	121 for 15 min	70 (65-75)
pH	3	62 (57-67)
	4 to 7	100
	8	65 (62-68)
	9	43 (38-48)
Enzymes	Protease	Negligible
	Trypsin	Negligible
	Amylase	100
	Lipase	100
	Catalase	100

[a]The values are means of 3 independent experiments performed in duplicates; the range is given in parenthesis. The untreated bacteriocin is having 1800 AU/ml of antimicrobial activity.

The bacteriocin inhibited *Listeria monocytogenes, Escherichia coli*, *Salmonella typhimurium*, *Bacillus* and *Aeromonas* organisms. Broad spectrum of antimicrobial activity of the bacteriocin may have its potential use as a food additive/preservative in food industry, particularly processed and refrigerated foods in which some *Bacillus* sps and *Listeria* are potential spoilage organisms. The antimicrobial compound produced from this strain can be characterized as bacteriocin, because inhibition due to acids and hydrogen peroxide had been excluded. Also the proteinaceous nature of the pediocin was confirmed by its sensitivity to proteolytic enzymes. These results were obtained with fairly pure preparation of pediocin obtained through cell adsorption-desorption and GPC, where in the probable role of low molecular weight compounds such as organic acids and hydrogen peroxide has been eliminated. The antimicrobial spectrum of the pediocin is in agreement with earlier reports (Kim et al, 2000; Jamuna et al, 2004; Vijai et al, 2010).

The biochemical properties of bacteriocin from this strain were similar to those of other bacteriocins from *Pediococcus* belonging to the Group II bacteriocins with respect to their molecular weight, heat and pH stability and sensitivity to proteolytic enzymes. The purified bacteriocin showed a single band of MW ~ 5.5 kDa which is in agreement with earlier reports (Jamuna et al, 2004; Kim et al, 2000).

The pediocin completely lost its antimicrobial activity after proteolytic treatment but retained its activity even after high temperature treatment and was stable at pH 3.0 to 7.0. Similar biochemical properties were observed for

bacteriocins produced by LAB such as pediocin PA-1/Ach, pediocin A (Bhunia et al, 1988; Ray et al, 2000).

Bacteriocins of several LAB are heat stable although it is lost after purification in some cases (Barefoot and Klaenhammer 1984). Heat stability character is advantageous if the bacteriocin is to be used as food preservative, since many food processing procedures involve heating steps. In addition bacteriocin activity at neutral pH makes an advantage over other bacteriocins used such as food preservatives and particularly over nisin, whose maximum solubility and stability are at pH 2 with these parameters decreasing significantly as the pH increases (Hurst 1981).

In conclusion the present study demonstrated the production of bacteriocin by *Pediococcus acidilactici*. The pediocin appeared to belong to Class II group of bacteriocins. Its broad spectrum of activity, temperature and pH stability suggested its potential applications in thermally processed and non-fermented foods.

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